MUCOSAL DEFENCE MECHANISMS

AGAINST

Nippostrongylus brasiliensis

A thesis submitted for the degree of

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by

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STATEMENT

Cell fractionation procedures were done in collaboration with Dr C.R. Parish (Department of Microbiology, JCSMR). The histological sections were cut and stained by Miss W. Hughes and Mr K. Mulgrue and electron microscopic observations were made by Dr H.R.P. Miller (Department of Immunology, JCSMR). With these exceptions the experiments described in this thesis were done by myself.

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SUMMARY

The effector mechanisms which bring about the expulsion of the intestinal parasite *Nippostrongylus brasiliensis (N. brasiliensis)* were investigated in rats with the aim of studying some general aspects of mucosal immunity. To this end, the worm burden kinetics in the recipients of immune thoracic duct lymphocytes (TDL) were examined and were related to the mucosal changes associated with the altered kinetics of parasite expulsion.

TDL drained from donor rats on the tenth day of a primary infection (day 10 TDL) conferred more protection against the adult parasite than any other source of immune cells. There was a direct and inverse relationship between the number of cells transferred and the number of parasites remaining in the intestine. Dose-response experiments confirmed that day 10 TDL were more effective than TDL drained from hyperimmune donors. These day 10 TDL conferred a high degree of protection not only against adult worms established by larval infection, but also against intraduodenally implanted 'normal' and 'damaged' worms.

When immune TDL were separated into populations of cells either lacking (sIg^-) or bearing surface immunoglobulin (sIg^+) by a rosetting procedure, protection was conferred only by the sIg^- population from day 10 TDL and predominantly by the sIg^- population from hyperimmune TDL. However, a small but significant degree of protection was also conferred by the sIg^+ population from hyperimmune TDL. The protective capacities of the unfractionated and of the separated and reconstituted hyperimmune TDL were greater than those of either subpopulation alone.

The hypothesis that a local anaphylactic shock in the intestinal mucosa enhances the passage of anti-worm antibody into the gut lumen was re-examined and the following aspects were studied: (a) the intestinal mast cell (IMC) response (b) specific IgE antibody levels in the serum (c) intestinal mucosal permeability.

There were considerable differences in the kinetics of worm expulsion and of the IMC in different strains of rats but in all instances significant increases in the numbers of IMC coincided with the final stage of the rapid phase of worm expulsion.

Increases in the numbers of IMC occurred in the infected recipients of immune TDL. The cells which conferred the response most effectively were those drained from donors 10 days after a primary infection. A relationship was observed between the numbers of IMC appearing in the mucosa and the dose of day 10 TDL transferred. When immune TDL were fractionated, only the cells lacking sIg were able to transfer the IMC response. Mast cells did not increase in normal recipients of day 10 TDL, suggesting that antigenic stimulation was required for their differentiation.

In agreement with several published reports, specific IgE antibody during primary infections was not detected until after the parasites had been expelled. Moreover, specific IgE 'memory-type' responses could be adoptively transferred by hyperimmune TDL but not by day 10 TDL. The magnitude of the IgE antibody responses in the recipients of hyperimmune TDL was directly related to the number of cells transferred. When hyperimmune TDL were fractionated into sIg^- and sIg^+ subpopulations, neither subpopulation alone transferred the specific IgE antibody response. However, the reconstituted population was able to confer an IgE response as effectively as the unfractionated hyperimmune TDL.

There was a marked increase in mucosal permeability during infection with *N. brasiliensis*. This pathological change was a function of the worm burden and was not related to the rapid phase of worm expulsion, to the rise in numbers of intestinal mast cells, or to any increase in the circulating IgE antibody titres.

The number of cells synthesizing IgA in the intestinal mucosa was also examined. Infection with 4000 L_3 caused a severe depletion of IgA-synthesizing cells in the lamina propria of the small intestine but the numbers rose to normal levels just prior to worm expulsion. On the other hand, infection with 1000 L_3 had little or no effect on the number of IgA-synthesizing cells in the intestine. IgA-synthesizing cells in the lamina propria of the small intestine of rats infected with *N. brasiliensis* were increased in number after the adoptive transfer of hyperimmune but not day 10 TDL. Fractionation of these hyperimmune TDL revealed that the sIg^+ subpopulation was responsible for this increase.

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

The structural integrity of the intestinal tract is closely related to its function both in terms of energy uptake and of protection against noxious stimuli. Even at the lower levels of evolution we can distinguish two basic functional cell layers (ectoderm and entoderm) with independent mobile phagocytic cells between the two layers. With the progess of evolution the structure and function of animals became more complicated; thus, the new cell types arising between ectoderm and entoderm begin to take a more dominant specialized role and are called mesoderm (Burnet, 1962).

The entoderm gives rise to the lining of the digestive tract and is primarily concerned with digestion and absorption. It also acts as a physico-chemical barrier against the outer environment. The mesodermal layer which, early in evolution, is represented only by a few wandering phagocytes, eventually gives rise to various specialized tissues including the lamina propria of mucosal surfaces which contribute to structural support, vascular supply, excretion, reproduction and defence reactions.

In vertebrates, the defence system of mesodermal origin has become highly specialized (the immune system) and includes a variety of cells such as plasma cells, lymphocytes, granulocytes and macrophages (Burnet, 1962). Because these cells are anatomically separated from the outer environment it is likely that an element of functional collaboration is required of the epithelial cells before the cells in the lamina propria can provide an effective barrier against infection.

GENERAL DEFENCE MECHANISMS IN THE INTESTINAL TRACT

1. Cellular components

The cells involved in the defence mechanisms of the intestinal tract can, as mentioned previously, be divided into two categories depending on

their origin. The epithelial lining and glandular epithelia of the intestine are derived from the entoderm, whereas the mesodermal layer gives rise to the muscular, vascular and connective tissues including free floating cells in the lamina propria, and to the serosal elements.

1.1 Epithelium

The cells of the intestinal epithelium are highly differentiated and are endowed with complex morphological specializations which integrate and translate the many stimuli into the cellular absorptive, secretory and excretory processes required to maintain homeostasis in the living organism.

The surfaces of the villi are covered by absorptive cells, goblet cells and a few entero-endocrine cells and the major role of the villi is to provide an increased surface area for absorption. Undifferentiated cells, immature goblet cells, entero-endocrine cells and Paneth cells line the crypts, and the major functions of the crypts are those of secretion and cellular proliferation. The undifferentiated cells proliferate actively and differentiate into absorptive, goblet, entero-endocrine and Paneth cells thus replacing effete cells lost from the villous tips (Leblond and Messier, 1958; Creamer, Shorter and Bamforth, 1961).

By the nature of the intercellular junctions between the epithelial cells, the epithelium presents a relatively impermeable barrier to macromolecules and to microorganisms. In addition, the surfaces of intestinal epithelial cells are physico-chemically protected by the mucus secreted by goblet cells and by the glycocalyx synthesized by the particular epithelial cells on which it is found (Ito and Revel, 1964; Ito, 1964). This protective coat has also been called 'antiseptic paint' (Heremans and Crabbé, 1967). Mucus has been reported to have parasiticidal (Frick and Ackert, 1948) and bactericidal (Jawetz, Melnick and Adelberg, 1958) activity, although these functions might, to some extent, be attributed to other

factors such as lysozymes, peroxidases, lactoferrin, α_1-antitrypsin, and complement which may be incorporated in the mucus (Newhouse, Sanchis and Bienenstock, 1976). A recent preliminary report (Bloch and Walker, 1977) suggests that mucus synthesis is increased in the presence of immune complexes.

Paneth cells, which lie at the base of the crypts of Lieberkühn, are among the most puzzling of the intestinal epithelial cells. Their distribution among different mammalian species is quite variable (Wheeler and Wheeler, 1964). They contain large quantities of lysozyme (Spicer, Frayser, Virella and Hall, 1977) which has a strong bacteriolytic activity (Fleming, 1922) and recently Erlandsen, Rodning, Montera, Parsons and Lewis (1976) found IgA to be present in Paneth cells. Intestinal epithelial cells, as well as respiratory epithelial cells, are capable of transporting luminal material including carbohydrate, protein, viruses, bacteria and fat across the cell towards the basement membrane (Bienenstock, 1974) and at least some of the ingested materials are sequestered into phagolysosomes (Straus, 1969). Furthermore, intestinal epithelial cells synthesize secretory component (SC) (Tomasi, Tan, Solomon, and Prendergast, 1965; Brandtzaeg, 1973, 1974) and contribute to the integration and transport of IgA and IgM (Rev. by Brandtzaeg, 1973).

Goblet cells are interspersed amongst the absorptive cells and secrete mucin, a high molecular weight glycoprotein which is rich in carbohydrate and is resistant to proteolytic enzymes (Forstner, Taichman, Kalnins and Forstner, 1973) and which, with water, forms a lubricating solution called mucus. The latter serves not only as a lubricant on the surface of the epithelium but also as a binding mechanism for secreted IgA on the epithelial surface by protein-protein interaction between their cysteine residues (Heremans, 1975). Since epithelial surfaces have phagocytic activities, Erlandsen

(1972) has proposed that epithelial cells may be able to phagocytose IgA-coated microorganisms or immune complexes containing IgA.

The other epithelial cell types are the gut endocrine cells of which eleven different types have been recognized (Solcia, Capella, Buffo and Frigerio, 1976). Whilst the enterochromaffin cell is the most easily recognized of these because of its content of 5-HT (Ersparmer and Asero, 1952; Benditt and Wong, 1957) the other entero-endocrine-like cells have been identified by immunofluorescence and by electron microscopy. They contain a variety of polypeptides which include gastrin, motilin, secretin, cholecystokinin and vasoactive intestinal peptide (Solcia *et al.* 1976). The functions of these polypeptide hormones in the gut have yet to be established but they probably serve a regulatory role. The origin of these cells is controversial with Pearse (1976) suggesting that they are derived from neural crest whereas other workers have proposed that they are of epithelial origin (Cheng and Leblond, 1974a, b).

The intestinal mucosal epithelium contains large numbers of mononuclear cells which have been called theliolymphocytes (Fichtelius, 1968). The origin and life-span of such cells remain controversial although they are reduced in number in thymectomized (Fichtelius, Yunis and Good, 1968, Ferguson and Parrott, 1972) and in antigen-deprived (Ferguson and Parrott, 1972) animals. Their protective functions have not been determined although it has been suggested that they may cause epithelial cell damage and, thus alter villus morphology (Ferguson and Jarrett, 1975).

1.2 Lamina propria

The intestinal lamina propria is a specialized type of connective tissue which contains a stroma of argyrophil fibres similar to that of lymphatic tissues (Bloom and Fawcett, 1968). The meshes of the argyrophil framework contain a variety of cell types amongst which lymphoid cells are predominant.

Lymphocytes in all stages of development are abundant in the intestinal lamina propria (Bloom and Fawcett, 1968; Deane, 1964). Both thymus-derived (T) (Guy-Grand, Griscelli and Vassali, 1974) and bone marrow-derived (B) (Ferguson and Parrott, 1972) lymphocytes are found in the intestinal lamina propria and also in the epithelial layer (thelio-lymphocytes).

Gowans and Knight (1964) first demonstrated that large dividing lymphocytes in rat thoracic duct lymph tended to localize in the gut-associated lymphoid tissue and this observation has been supported by numerous subsequent studies (Griscelli, Vassali and McCluskey, 1969; Hall, Parry and Smith, 1972; Halstead and Hall, 1972; Parrott and Ferguson, 1974; Guy-Grand, *et al*. 1974). Guy-Grand *et al*. (1974) showed that B- and T-blasts from mesenteric lymph node and thoracic duct lymph have a marked tendency to localize in the gut. They also suggested that gut-homing blasts are the progenitors of the intestinal IgA plasma cells and that gut-homing T-blasts preferentially migrate into the intestinal epithelium.

The functions of T-lymphocytes in the gut are not known. Recently Rose, Parrott and Bruce (1976a,b) demonstrated a selective accumulation of T-blasts in the mucosa of parasitised small intestine or in oxazolone-sensitized skin. This would suggest that T-lymphocytes are involved in local cell mediated responses along the intestinal tract. In this regard, Ferguson and Jarrett (1975) have proposed that T-lymphocytes may be involved in the generation of villus atrophy which occurs during parasitic infections. Lymphocytes, as well as plasma cells, are relatively sparse in the lamina propria of germ-free animals (Crabbé, Nash, Bazin, Eyssen and Heremans, 1970) which would suggest that antigen may have some influence on the development and structure of gut-associated lymphoid tissue.

Although various classes (IgG, IgM and IgA) of immunoglobulin-synthesising cells are found in the intestinal lamina propria in conventional animals of most species including man, plasma cells producing IgA are predominant in this site (Crabbé and Heremans, 1966; Nash, Vaerman, Bazin and Heremans, 1969; Crabbé *et al.*, 1970). Tada and Ishizaka (1970) reported that IgE-synthesizing plasma cells are primarily located along the respiratory and gastrointestinal mucosae in primates, although more recent studies in the rat suggest that the cells in the mucosa which contain IgE are mast cells (Mayrhofer, Bazin and Gowans, 1976).

Eosinophils are the predominant type of granulocyte in the lamina propria (Deane, 1964; Bloom and Fawcett, 1968). Localized accumulations of eosinophils are found following primary antigenic stimuli and a 'memory'-type response can be induced by a secondary challenge with the same antigen (Rev. by Speirs, 1970).

Although eosinophils are found in association with various pathological conditions such as parasitic infections, hypersensitivity responses, malignant tumors and graft rejection (Rev. by Speirs, 1970) their functions are not fully understood. They have, however, been shown to kill antibody-coated schistosomulae *in vitro* (Butterworth, Sturrock, Houba, Mahmoud, Sher and Rees, 1975).

The tendency for eosinophils to accumulate beneath surfaces exposed to the environment such as skin, bronchus and gut strongly suggests that they have an important function in these areas (Rev. by Hirsh, 1965).

The eosinophil response is, to some degree, regulated by lymphocytes (Basten and Beeson, 1970), although eosinophilotaxis may also be induced by non-immunological stimuli (Rev. by Speirs, Speirs and Ponzio, 1974; Archer, Robson and Thompson, 1977). Eosinophilopoiesis and eosinophilotaxis are also influenced by histamine (Rev. by Archer, 1970).

The release of eosinophil chemotactic factor of anaphylaxis (ECF-A) from sensitised mast cells upon contact with antigen will also cause eosinophil migration (Wasserman, Goetzl and Austen, 1974).

Free floating phagocytic cells form an important line of defence beneath mucosal epithelia. Foreign substances, which have gained access to the lamina propria may be phagocytosed and digested by macrophages. This may, in turn, lead to the development of an immune response within the mucosa and draining lymph nodes. The role of macrophages during the immune response *in vivo* and *in vitro* has been widely reviewed (Argyris, 1974).

Intestinal mast cells (IMC) are commonly found in the lamina propria in rodents (Enerbäck, 1966a) and are morphologically and histochemically different from the connective tissue mast cells (Enerbäck, 1966a,b,d; Miller and Walshaw, 1972). They are derived from lymphoblastoid cells, which undergo cell division and differentiation *in situ* (Miller, 1969, 1971a; Miller and Jarrett, 1971). The proliferating IMC degranulate and migrate into the intestinal epithelial layer to become globule leucocytes during parasitic infections (Miller, Murray and Jarrett, 1967; Murray, Miller and Jarrett, 1968; Miller, 1971a; Miller and Walshaw, 1972). Athymic (*nu/nu*) mice were reported to have few IMC after infection with *N. brasiliensis* although the number of connective tissue mast cells was normal (Olson and Levy, 1976) and these nude mice were able to mount a passive cutaneous anaphylaxis (PCA) response (Keller, Hess and Riley, 1976). The stimuli which cause mast cell hyperplasia in normal rats during parasitic infection have not been identified although Archer *et al.* (1977) have shown that lipid extracts from ascaris will, after intraperitoneal injection, cause a rise in mast cell numbers.

Mast cells contain several biologically active substances such as heparin, histamine and 5-HT (Rev. by Csaba and Nilzén, 1974). They have receptors for IgE on their surface and, after the interaction of antigen with mast cell-bound IgE they release chemical mediators (Rev. by Ishizaka and Ishizaka, 1975). The interaction of allergen with IgE on the mast cell surface has, therefore, an important role in the pathogenesis of a variety of allergic conditions (Rev. by Lichtenstein, 1972). The possible role of intestinal mast cells in protection against *N. brasiliensis* will be discussed in detail later in this chapter.

2. Antibodies in the Intestinal Tract

Although Davis, in 1922, demonstrated specific coproantibody in saline extracts prepared from the faeces of patients suffering from dysentery, Besredka (1927) was the first to postulate that local immunity could be established independently of systemic immunity. There have been several subsequent reports describing the presence of copro-antibodies directed against a variety of pathogenic bacteria (Rev. by Pierce, 1959). The earliest experiments designed to determine the importance of local antibody were reported by Burrows and coworkers (Rev. by Pierce, 1959). However, relatively little attention was given to the concept of a local secretory immune system until Heremans, Heremans and Schultze (1959) isolated and purified the IgA class of antibodies in serum. Since then, there has been extensive research into the secretory IgA system and this has been reviewed on several occasions (Tomasi and Bienenstock, 1968; Heremans, 1968; Tomasi and Grey, 1972; Brandtzaeg, 1973; Heremans, 1974; Bienenstock, 1974) and has been the subject of several published proceedings (Dayton, Small, Chanock, Kaufman and Tomasi, 1969; Mestecky and Lawton, 1973).

All immunoglobulin classes are found in the intestinal lumen and, consistent with the predominance of IgA plasma cells in the mucosa, IgA is the principal class of immunoglobulin in the lumen (Chodirker and Tomasi, 1963; Heremans and Crabbé, 1967; Tomasi and Bienenstock, 1968; Vaerman and Heremans, 1968; Tomasi and Grey, 1972; Heremans, 1974). Moreover, after oral immunization with various antigens, specific IgA antibodies can be detected in the intestinal secretions (Ogra, Karzon, Righthand and MacGillivray, 1968; Shearman, Parkin and McClelland, 1972).

Pierce (1976) stated: "The prevention of enteric infections by immunization has been attempted for nearly 85 years. Yet, with the important exception of oral immunization with live attenuated poliovirus, none of these attempts has been highly successful. This may be because all present enteric vaccines were developed when understanding of the immune mechanisms operative in the gut was very limited". He suggested that parenteral, followed by oral challenge might be an effective means of immunizing the intestine with non-replicating protein antigens (Pierce, 1976).

Secretory IgM appears to be synthesized principally in the plasma cells of the adjacent mucosa (Brandtzaeg, Fjellanger and Gjeruldsen, 1968) and is then selectively transported across the epithelium (Brandtzaeg, 1973, 1975; Brown, Isobe and Nakane, 1976).

IgG is found in the intestinal fluid, but very few discrete IgG plasma cells are seen in the lamina propria of most species (Vaerman and Heremans, 1969). Thus, IgG probably reaches the gut lumen either as a result of transudation (Tomasi and Grey, 1972) or via the bile duct (Fukumoto and Brandon, 1977).

Brandtzaeg (1973) has proposed two lines of defence with respect to local immune responses. The first line of defence is represented by secretory IgA and, to a lesser extent, by 19S IgM which furnishes the mucous coat with protective antibodies. The proposed second line of defence is represented by complement-fixing IgG antibodies which are supplied to the local site by extravasation.

Tada and Ishizaka (1970) reported that IgE-synthesizing plasma cells are primarily found along the respiratory and gastrointestinal mucosae in primates. Fragments of IgE have been detected in human intestinal fluid (Brown, Borthistle and Chen, 1975; Brown and Lee, 1976) and IgE has been detected in nasal fluid and sputum of asthmatic patients (Ishizaka, Ishizaka, Tada and Newcomb, 1969). From these observations, it has been proposed that increased permeability caused by the interaction of IgE antibody with antigen might cause a leak of immunoglobulin into the area and contribute to local protection (Ishizaka *et al.*, 1969).

In general, although the protective activity of IgA antibodies in the intestinal fluid is well documented (Rev. Tomasi and Grey, 1972), the roles of other immunoglobulin classes in the gut lumen are poorly understood.

3. Hypersensitivity Reactions in the Intestinal Tract

Hypersensitivity reactions can be divided into four types (Coombs and Gell, 1963):

Type I : anaphylactic, reagin dependent

II : cytotoxic or cell stimulating

III : damage by antigen-antibody complex, Arthus type

IV : delayed, Tuberculin type.

Type I-III are immediate hypersensitivity reactions, and require serum antibody. Type IV can be adoptively transferred by thymus

derived lymphocytes and is called cell-mediated immunity. These hypersensitivity reactions cause severe systemic and/or local pathological changes. There is, however, great species variation in the mechanism of anaphylaxis in animals (Austen and Humphrey, 1963). Similarly, in an anaphylactic response the 'shock organs' and the functions of pharmacological mediators vary according to the species affected (Austen and Humphrey, 1963). For example, in rats the 'shock organ' during anaphylaxis is the intestine and major mediator is 5-HT, whereas in guinea pigs the lungs are the primary target and the mediator is histamine (Austen and Humphrey, 1963).

The Arthus-type response is characterized by increased vascular permeability and infiltration of polymorphonuclear leucocytes at the site of antigen challenge in sensitized animals (Rev. by Cochrane, 1965). There are relatively few reports of Arthus-type responses in the gut, although Bellamy and Nielsen (1974) demonstrated in pigs sensitized to bovine serum albumin (BSA), that exposure of the intestinal mucosa to BSA induced antigen-specific emigration of large number of neutrophils into the intestinal lumen. There is a possibility that these cells either have a phagocytic function in the gut lumen or, alternatively, that they are degraded and release their lysosomal contents which would have an anti-bacterial activity (Baggiolini, 1972).

Examples of delayed type hypersensitivity (DTH) in the gut have been reported more frequently in the literature, thus, guinea pigs sensitized cutaneously with dinitrochlorobenzene develop lesions of DTH in the colon after repeated rectal challenge with this chemical (Bicks and Rosenberg, 1964). Delayed responses may be implicated in the mucosal lesions associated with coeliac disease, since gliadin induced production of migration inhibitory factor occurs in human jejunal biopsy specimens from patients suffering from this disease (Ferguson, MacDonald, McClure

and Holden, 1975). These results suggest that cell-mediated immune responses may be involved in some pathological conditions of the intestinal tract.

At the moment, there is no clear-cut evidence that hypersensitivity reactions in the gut have any beneficial effects, although Urquhart and co-workers (Urquhart, Mulligan, Eadie and Jennings, 1965) have proposed that local anaphylaxis may have a protective role against *N. brasiliensis* in the rat. This proposition will be discussed in detail later in this chapter.

4. Influence of the Thymus

The role of T-lymphocytes in the immune response has been a topic of intensive research for a number of years and has been extensively reviewed (e.g. Craddock, Longmire and McMillan, 1971). T-cells are responsible for cell-mediated immunity (Rev. by Cooper and Lawton, 1974) and also regulate B-cell function ('help' and 'suppression') in the production of antibodies (Rev. by Katz, 1974; Mitchell, 1974). Thymus-dependency of IgA production has been reviewed by Lamm (1976). Furthermore, a variety of cells in the mucosa, such as mast cells eosinophils and intestinal epithelial cells are, to some extent, under the influence of the thymus (see this chapter and also chapter 5). One characteristic clinical manifestation of "post-thymectomy wasting syndrome" is severe diarrhoea (McIntire, Sell and Miller, 1964; Good and Gabrielsen, 1964), and similar observations have been made in athymic (*nu/nu*) mice raised under conventional conditions (Rev. by Rygaard, 1973). Since the wasting syndrome of neonatally thymectomized or athymic mice can be prevented by thymus transplants, there is little or no doubt that the thymus in some way regulates the local defence mechanisms along the intestinal tract.

5. Peyer's Patches and Intestinal Immunity

Peyer's patches are unique aggregates of lymphoid tissue present in the small intestines of mammals. Because of their location and because there is an intimate relationship between lymphoid and epithelial structures within them, they have been regarded as the mammalian equivalent of the avian bursa of Fabricius (Good and Finstad, 1967) although they contain both T- and B-cells (Raff and Owen, 1971 Waksman, 1973; Kagnoff and Campbell, 1974; McWilliams, Phillips-Quagliata and Lamm, 1975).

The functions of Peyer's patches in local defence mechanisms have been summarized and discussed in detail by Waksman and Ozer (1976). Thus, after being primed by antigens from the gut lumen, both T- and B-lymphocytes leave Peyer's patches and enter the systemic circulation via the mesenteric lymph nodes and thoracic duct. The majority of T-lymphocytes relocalize in the intestinal mucosa where they may migrate into the surface epithelium and be involved in local cell-mediated immune responses. Antigen-primed B-lymphocytes leaving Peyer's patches can be subdivided into several subpopulations: (1) A population which has membrane-bound IgA (sIgA) and localizes in the lamina propria (Rev. by Lamm, 1976; Rev. by Cebra, Craig and Jones, 1976); (2) A population which contributes to a major part of the total immunocompetent surface IgM-bearing lymphocyte pool in the spleen and lymph node (Armstrong, Diener and Shellam, 1969) and which provides memory B-cells for systemic IgM responses (Cooper and Turner, 1969); (3) Some B lymphocytes which may differentiate into IgE-forming cells (Tada and Ishizaka, 1970).

These findings strongly suggest that Peyer's patches themselves have little or no local protective function, but that they disseminate lymphocytes which contribute to systemic and to local immune responses. "The central element of this specialized function is the switch of surface

immunoglobulin on the surface of immunocompetent B cells bearing IgM to IgA or IgE under the influence of unknown structural elements of gut-associated lymphoid tissue itself" (Waksman and Ozer, 1976).

NIPPOSTRONGYLUS BRASILIENSIS AS AN EXPERIMENTAL MODEL

Since it was first described by Travassos (1914) and Yokogawa (1920), *N. brasiliensis*, a nematode which parasitises the small intestine of the rat, has been widely used as a laboratory model in studies of helminth immunity, of allergic reactions, and of pathological conditions of the intestine (Rev. by Ogilvie and Jones, 1971). The life cycle of this parasite was first described by Yokogawa (1920) and its general biology has been studied in detail by Haley (1961, 1962).

When infective third-stage larvae (L_3) are injected subcutaneously into rats, they travel via the blood stream to the lungs, where they moult to become the fourth larval stage (L_4). They then migrate up the trachea, are swallowed, and pass through the oesophagus and stomach. The fourth larval stages parasitize the anterior third of the small intestine where they undergo a further moult to become adult worms (Rev. by Ogilvie and Jones, 1973).

The main advantages of using *N. brasiliensis* to study mucosal defence mechanisms are as follows: the parasites do not multiply within the host as do live bacteria and viruses; stable populations of adult worms are established and are harboured in the intestine after a single subcutaneous inoculation of L_3 (Jarrett and Urquhart, 1971); adult worms live on the surface of the epithelium, intertwined amongst the villi of the small intestine and they do not penetrate the epithelial layer (Barth, Jarrett and Urquhart, 1966; Ogilvie, 1974; Symons, 1976); the immune status of the host is readily measured by counting the worm burden or by monitoring eggs in the faeces; a wide range of host responses, such as systemic and/or

local antibody production, hypersensitivity reactions and inflammatory responses, are provoked during infection (Rev. by Ogilvie and Jones, 1971, 1973).

Since Taliaferro and Sarles (1939) described details of the cellular reactions in *N. brasiliensis*-infected rats, there have been many studies on the contributions of humoral and of cellular components in the immune response against *N. brasiliensis*. These are reviewed by Jarrett and Urquhart (1971), Ogilvie and Jones (1971, 1973), Murray (1972), Kelly (1973) and Ogilvie and Love (1974).

ANTIGENS OF *N. BRASILIENSIS*

During the different developmental stages of the parasite, a large number and complexity of antigenic materials of somatic and of metabolic origin may be released. No protective antigens have been characterized or even isolated as a single component, although an allergen has been partially purified from adult worm extracts (Hogarth-Scott, 1967; Wilson, 1967; Jones and Ogilvie, 1967; Ambler and Orr, 1972). However, its ability to protect against infection has not been studied in detail.

Prochazka and Mulligan (1965) failed to demonstrate protective activity by infecting rats with X-ray 'inactivated' larvae even though the latter could be found in the lungs for 10 days after infection (Jennings, Mulligan and Urquhart, 1963). In contrast, fewer than 10 adult female worms stimulated a strong resistance to reinfection (Ogilvie, 1965a). It would appear from these observations that the major source of protective antigen is the adult worm.

Some protection has been reported in rats immunized intraperitoneally with culture fluids from infective larvae (Thorson, 1953) or from adult worms, together with pertussis vaccine (Denham, 1968, 1969). On the other hand, intraperitoneal injection of worm extracts and pertussis vaccine (Ogilvie, 1967) had no protective effect, which would suggest that

the antigens which cause the protective immune response of the host are actively produced and released during the metabolism of the parasites. Allergen has also been found in relatively large quantities in culture fluid from adult worms and is apparently actively released by live worms (Ogilvie and Jones, 1969). Recently Poulain, Pery and Luffau (1976b) demonstrated the potent immunogenicity of adult worm metabolites after examining the effects of oral immunization with worm extracts, with culture fluid from adult worms, and with killed worms. Protection was conferred to rats immunized with worm extracts or with culture fluids but not to rats immunized with dead worms. Their experiment also indicated that the route of immunization is of great importance in stimulating a protective response.

Quantitative and/or qualitative antigenic differences between larval and adult stages are suggested by the work of Love, Kelly and Dineen (1974) who found that syngeneic transfer of immune mesenteric lymph node cells caused expulsion of parasites from the intestine but failed to affect the migration of the pre-intestinal larval stages. Furthermore, Love (1975) demonstrated that transplanted fourth stage larvae are more susceptible to the combined action of transferred immune cells and immune serum than are transplanted adult worms.

THE IMMUNE RESPONSE AGAINST *N. BRASILIENSIS*

1. Active Immunity

Rats become relatively resistant to re-infection when they have recovered from a primary infection with *N. brasiliensis* (Africa, 1931; Sarles and Taliaferro, 1936). Sarles and Taliaferro (1936) demonstrated that the protective mechanisms against re-infection are expressed not only in the intestine but also in the lungs and, to a slight extent, in the skin. Analysis of the worm burden kinetics during primary, secondary and tertiary infections with 3000 L_3 (Jarrett, Jarrett and Urquhart, 1968a;

Jarrett and Urquhart, 1971) supports this concept. Similarly, Love, Kelly and Dineen (1974) have reported that larval migration is retarded in secondary and tertiary infections. Jarrett, Jarrett and Urquhart (1968a) subdivided the course of infection into 4 phases. Loss Phase 1 occurs at the site of injection or during the course of migration via the lungs to the intestine. Loss Phase 2 occurs after a Plateau Phase during which the intestinal worm burden remains stable. During Loss Phase 2 the worms are rapidly expelled from the gut lumen and, subsequently, a small residual population of worms survives in the intestine (Threshold Phase). A Threshold Phase is not, however, observed in all strains of rats (Chapter 3). These results (Jarrett *et al.*, 1968a) show that both the magnitude and the rate of each Loss Phase are increased after secondary and tertiary infections.

Taliaferro and Sarles (1939), after examining the cellular reactions in the skin, lungs and intestines of *Nippostrongylus*-infected normal and immune rats, suggested that the local cellular response is essentially the same in primary and repeated infections except that the intensity of the response is increased after re-infection. Yet there is at present, insufficient evidence to determine whether the protective mechanisms involved in the expulsion of adult worms are identical in primary and repeated infections.

2. 'Self-Cure'

This phenomenon was first described by Stoll (1929) who infected worm-free lambs with *Haemonchus contortus* and grazed them on a limited area of pasture where they would be subject to re-infection. He observed a sudden dramatic fall in the faecal egg counts which he thought was probably due to the elimination of adult worms. Similar phenomena have been observed with other nematode infections (Fernando, 1968; Denham, Ponnudurai, Nelson, Rogers and Guy, 1972). Stewart's series of experiments (Rev. by Stewart,

1959) provided strong evidence that 'self-cure' is due to an immediate-type hypersensitivity reaction.

Recently, the term 'self-cure' has been widely applied to describe the termination of a primary helminth infection (Mulligan, Urquhart, Jennings and Neilson, 1965; Urquhart, Mulligan, Eadie and Jennings, 1965; Mulligan, 1968; Smithers, 1972) and although the classical 'self-cure' phenomenon and the termination of a primary infection have many features in common (Mulligan, 1968), Ogilvie and Jones (1968) have pointed out that these phenomena might be mediated by quite different immunological mechanisms. The term 'self-cure' should, therefore, be used with care until the immunological mechanisms involved have been further clarified.

3. Analysis of the Immune Response to *N. brasiliensis*

3.1 Thymus dependency

Expulsion of *N. brasiliensis* from the intestine can be suppressed by neonatal thymectomy in rats (Ogilvie and Jones, 1967; Wilson, Jones and Leskowitz, 1967; Kelly, 1971), by anti-lymphocyte serum treatment in mice (Kassai, Szepes, Rethy and Toth, 1968) or by a combination of neonatal thymectomy and anti-thymocyte serum treatment (Kelly, 1972). Jacobson and Reed (1974) showed that congenitally hypothymic (*nu/nu*) mice failed to expel *N. brasiliensis*. They also demonstrated that the ability of these mice to expel worms could be restored by thymic implantation. Such results point to the influence of the thymus on the immune mechanisms involved in worm expulsion.

Since Miller (1961) first described neonatal thymectomy in mice, the function of thymus derived lymphocytes (T-lymphocytes) has been a major topic of research in immunology. The current view is that thymus-derived lymphocytes are not only involved in cell-mediated immune responses, such as graft rejection and DTH, but can also 'help' or 'suppress' antibody production (Rev. by Katz, 1974, Mitchell, 1974). Recent experiments by

Jacobson and Reed (1976) suggest that both the humoral and the cell-mediated steps in the expulsion of *N. brasiliensis* from mice are thymus dependent. It is not clear whether this is also true of the rat.

3.2 Transfer of protective activity

The relative roles of humoral antibody and of cell-mediated immune responses in protection against *N. brasiliensis* have been assessed by the transfer of immune serum and/or lymphoid cells. To ensure that the immune responses were directed against adult worms within the intestine, the parasites were, in many instances, implanted intraduodenally. The results of these experiments have been extensively reviewed (Jarrett and Urquhart, 1971; Ogilvie and Jones, 1971, 1973; Kelly, 1973; Ogilvie and Love, 1974).

The first attempt to passively immunize rats against *N. brasiliensis* was reported by Sarles and Taliaferro (1936). Various workers (Chandler, 1937, 1938; Sarles, 1939; Mulligan *et al.*, 1965; Jones and Ogilvie, 1967b; Ogilvie and Jones, 1968) were also able to transfer protective activity with immune serum. However, it is difficult to compare these different results because the strain of rats used, the schedule for immunizing the serum donors, the timing and the dose of serum transfer, and the dose of infective larvae given to recipients were different in each experiment. In general, protection conferred by passive transfer of immune serum was variable and seldom comparable to that produced by active infection.

Good evidence for the role of antibodies in the expulsion of adult worms was provided by Ogilvie and co-workers who used adult worm implantation and the transfer of immune serum (Ogilvie and Hockley, 1968; Jones and Ogilvie, 1971). The results of these experiments were as follows: when adult worms harvested from donors after day 10 of a primary infection ('damaged' worms) were implanted intraduodenally into normal rats, they were expelled by day 6 (Ogilvie and Hockley, 1968). Adult worms obtained from

donors 6-9 days after primary infection ('normal' worms) and implanted by the same route into normal rats, were not expelled for at least 8 days (Jones and Ogilvie, 1971). However, when 'normal' worms were implanted into recipients treated with immune serum, they were expelled by day 6. Similarly, when worms were recovered 2 days after being implanted into rats treated with immune serum and were re-implanted into normal rats, they were also expelled by day 6 (Jones and Ogilvie, 1971). On the basis of these results it has been proposed that antibody damages the worms and that this damage is a necessary step before other non-specific mechanisms to effect worm expulsion. This non-specific mechanism would appear to be radiosensitive but resistant to anti-lymphocyte serum (Jones and Ogilvie, 1971).

Barth *et al.* (1966) proposed that a local anaphylactic response might cause increased mucosal permeability, thus enhancing the transport of antibody into the gut lumen. The arguments for and against this proposition have been reviewed (Jarrett and Urquhart, 1971; Ogilvie and Jones, 1971; Murray, 1972; Ogilvie and Love, 1974) and will be discussed later in this Chapter.

Neonatal rats, which normally expel their worm burden more slowly than adults (Jarrett, Jarrett and Urquhart, 1968b; Ogilvie and Jones, 1967), can be passively immunized by sucking immune mothers (Jones and Ogilvie, 1967b; Greenberg, 1971) or by feeding them with immune serum (Jones and Ogilvie, 1967b). Interestingly enough, adult rats are themselves unable to expel worms during lactation (Connan, 1970; Dineen and Kelly, 1973). Experiments with prolactin suggest that the failure of lactating rats to expel worms is under hormonal influence (Kelly and Dineen, 1973).

The adoptive transfer of resistance against *N. brasiliensis* with lymphoid cells was first attempted by Hunter and Leigh (1961). They transferred a mixed cell population pooled from lymph nodes and spleen intraperitoneally, into recipients which were infected with L_3 4-5 days after

cell transfer, but these experiments were unsuccessful. Ogilvie and Jones (1968) re-investigated the adoptive transfer of protection against the parasite with immune cells. They transferred a mixed cell population pooled from the peritoneal cavity, lymph nodes and bone marrow by various routes into irradiated recipients which were infected with L_3 on the day of, or 6 days after, cell transfer; but again their results were variable and only 3 out of 11 batches of cells were protective. Similarly, Kassai and Szepes (1970) transferred a mixed cell population from spleen, mesenteric lymph node and bone marrow intravenously into irradiated recipients which were infected with 1000 L_3,and protection was assessed by faecal egg counts and by worm counts 21 days after infection. These experiments also failed to adoptively immunize against *N. brasiliensis*.

These results suggest that the source of the cells, the route of cell transfer, the condition (i.e. normal or irradiated) of the recipients and the time after infection that the cells are transferred could be important factors in the success of such experiments.

The observation that transplanted 'damaged' worms were rapidly expelled from normal recipients but not from irradiated recipients (Ogilvie and Jones, 1968; Ogilvie and Hockley, 1968; Jones and Ogilvie, 1971) led Ogilvie and co-workers to propose that antibody damage is a pre-requisite for the non-specific, radiosensitive expulsive mechanisms (Jones and Ogilvie, 1971; Rev. by Ogilvie and Jones, 1971). Subsequently, Keller and Keist (1972) demonstrated that expulsion of 'damaged' worms was inhibited in young rats and in irradiated (400 r) rats and that the expulsive mechanism could be restored by transfer of normal mesenteric lymph node cells but not by peritoneal exudate cells or mesenteric lymph node cells subjected to 5000 r prior to injection. From these observations they proposed that the lymphocyte is a major component in the non-specific expulsive mechanisms.

Kelly and Dineen (1972a)reported that transferring syngeneic immune mesenteric lymph node cells (MLNC) into recipients which were infected with L_3 at the same time, caused rapid expulsion of the parasites. In a subsequent experiment Dineen, Ogilvie and Kelly (1973b)demonstrated that syngeneically transferred immune MLNC caused the rapid and complete expulsion of transplanted 'damaged' worms but these cells were less effective against transplanted 'normal' worms. The protective role of immune MLNC was further examined by Dineen, Kelly and Love (1973a)who found that transplanted 'damaged' worms, or adult worms developed from larval infection both in irradiated (400 r) and in non-irradiated syngeneic recipients were rapidly expelled by the adoptive transfer of immune MLNC. On the other hand, they were unable to confirm the work of Keller and Kiest (1972) in which normal MLNC caused the expulsion of 'damaged' worms from irradiated (400 r) recipients.

In addition, Dineen and co-workers (Dineen and Kelly, 1973; Kelly, Dineen and Love, 1973) demonstrated that immune MLNC did not cause the expulsion of transplanted 'damaged' worms in heavily irradiated (750 rad) recipients, although these cells were able to cause worm expulsion in non-irradiated recipients and in recipients irradiated with 400 r. Heavily irradiated recipients were able to expel 'damaged' worms only when they were restored with both immune lymphocytes and bone marrow cells (Dineen and Kelly, 1973).

These observations have been summarized by Kelly (1973) and also by Ogilvie and Love (1974) and have led them to propose a 'multiphasic hypothesis' (Kelly, 1973) or 'two-step theory' (Ogilvie and Love, 1974) of worm expulsion. In the first step, specific antibodies cause irreversible worm damage thereby making them susceptible to the subsequent, lymphocyte-mediated, non-specific expulsive step. This non-specific step may

involve cell-mediated hypersensitivity and/or a myeloid cell response such as mast cells and eosinophils (Ogilvie and Love, 1974).

Recently, Ogilvie, Love, Jarra and Brown (1977) reported that the expulsion of transplanted 'damaged' worms from the intestines of heavily irradiated (750 rads) rats could be induced by immune thoracic duct lymphocytes (TDL) or MLNC. The effector cells appeared by the eighth day after infection and, after fractionation of the TDL, were found in the population of cells lacking surface immunoglobulin. These findings refute the proposal by Dineen and co-workers (Dineen and Kelly, 1973; Kelly *et al.*, 1973) that a bone marrow component is required for worm expulsion.

Although there is no doubt that lymphocytes have an important role in the expulsion of the adult parasites, the accumulated data cited above indicates that this role is far from being defined. Indeed, such variables as the source and dose of cells, the immunological status of the cell donor, the maturity of the parasites, the level of infection in, and the immunological status (irradiation, thymectomy, age, sex, pre-treatment with drugs etc.) of the recipients have never been systematically examined. Nor has there been any study of the kinetics of worm expulsion in adoptively immunized rats. Until the effects of some of these variables have been assessed the mechanisms involved in adoptive immunity will remain controversial.

3.3 Immunoglobulin classes involved in protection against *N. brasiliensis*

Four major classes of immunoglobulins (IgG, IgM, IgA, and IgE) and four subclasses of IgG (IgG_1, IgG_2a, IgG_2b, IgG_2c) have been defined in the rat by Bazin, Beckers and Querinjean (1971) who studied immunocytoma proteins.

The protective activity of the different immunoglobulin classes against *N. brasiliensis* has been examined by Jones and co-workers (Jones, Edwards and Ogilvie, 1970; Rev. by Ogilvie and Jones, 1971) who obtained

immune sera from rats after primary, secondary or tertiary infections with L_3 or after infection with transplanted adult worms. The antisera were fractionated by DEAE-cellulose chromatography or by Sephadex G200 gel filtration and were passively transferred into recipients which were infected with L_3 or with intraduodenally implanted 'normal' adult worms. Their results suggested that protective activity against *N. brasiliensis* is conferred by 7S γ_1 (= IgG_1). A slight protective effect was also occasionally detected in the γM (= IgM) and γA (= IgA) fractions. Antisera obtained after a tertiary infection had an additional 7S γ_2 (= IgG_2a?) antibody activity. They also found that protection could be passively transferred by fractions which had no PCA activity and concluded that IgE had no significant protective function against *N. brasiliensis*. This is at variance with the conclusion of Wilson and Bloch (1968) who emphasized the roles of IgE and mast cells in worm expulsion by demonstrating that peritoneal mast cells were sensitized with homocytotropic antibody as early as day 10 of a primary infection with *N. brasiliensis*. However, Keller (1970) was unable to confirm that peritoneal mast cells were sensitized at this stage of infection.

Precipitating antibody against *N. brasiliensis* was reported by Sarles and Taliaferro (1936) and by Sarles (1938) who, after incubating infective larvae with immune sera, observed that precipitates were formed around the anterior orifice and the excretory pore apertures of the parasites. In this regard Schwabe (1957) and Mulligan *et al.* (1965) described a decrease in oxygen consumption by third stage larvae after incubation in immune serum. These *in vitro* experiments suggest that antibody could have a protective activity against the parasites.

Precipitation reactions between extracts of adult worms or larvae and immune serum have also been reported (Ogilvie and Jones, 1971; Wilson, 1967). Recently Poulain, Luffau and Pery (1976a) found hemaggulutinating

antibody in the intestinal fluid 6 days after infecting rats with 3000 L_3. This antibody was identified as IgA by Biogel A 1.5M gel filtration. They also identified circulating hemaggulutinating antibodies of the IgM and IgG classes by Biogel A5M gel filtration. However the protective activities of such antibodies are not fully understood. These results must be interpreted with some caution because there are several reports of non-specific precipitation reactions between worm extracts and normal serum (Ogilvie and Jones, 1971) and between human intestinal fluid and heterologous antisera (Kraft, Rothberg and Kriebel, 1970).

3.4 Cells involved in protective immunity against *N. brasiliensis*

A detailed qualitative analysis of the cellular reactions in the skin, lungs and intestines of rats undergoing primary and secondary infections with *N. brasiliensis* has been described by Taliaferro and Sarles (1939). A mixed inflammatory reaction including plasma cells, lymphocytes, mononuclear cells, macrophages, eosinophils, basophils and, in particular, large numbers of connective tissue basophils (= mast cells) and globule leucocytes were observed at the height of the immune reaction to the parasites.

The kinetics of intestinal eosinophils in rats infected with *N. brasiliensis* was examined by Wells (1962) and also by Kelly and Ogilvie (1972). Their results showed that eosinophils rapidly increased in number near the time of the final stage of worm expulsion. The protective function of eosinophils is not clear and although Hogarth-Scott and Bingley (1971) demonstrated that an antiserum prepared against peritoneal exudate cells, suppressed worm expulsion, it is not clear whether this serum which had anti-eosinophil activity also had activity against other cell types.

Intestinal mast cell (IMC) kinetics were first examined by Wells (1962). The most extensive studies on this cell have been carried out by Miller and co-workers (Miller *et al.*, 1967; Miller, 1969; Murray, Jarrett and Jennings,

1971; Miller and Jarrett, 1971; Miller and Walshaw, 1972). The latter group proposed that IMC, which increased in number during worm expulsion, caused a rise in mucosal permeability, thus permitting the release of anti-worm antibody into the gut lumen. It was thought likely that the observed degranulation of IMC during worm expulsion was brought about by the interaction of worm antigen with IgE bound to the surface of the cells.

The role of IMC in worm expulsion has been extensively reviewed (Murray, 1972; Ogilvie and Love, 1974; Waksman and Ozer, 1976). However, a number of workers have been unable to correlate worm expulsion with the rise in mast cell numbers and have discounted any protective role for mast cells (Keller, 1971; Kelly and Ogilvie, 1972). Recently Ogilvie *et al.* (1977) were unable to find either IMC or eosinophil responses in irradiated rats (750 rad) which had received immune TDL and implanted 'damaged' worms. Moreover, they found that 'damaged' worms were expelled by non-immunoglobulin-bearing lymphocytes in the absence of other accessory cells. They did, however, point out that irradiation had severe effects on the intestinal epithelium such that the intestinal environment could have been altered and the susceptibility of the worms to the host's protective responses may have been modified.

Although the protective roles of mucosal plasma cells and lymphocytes in worm expulsion have not been studied it is reasonable to assume that a local IgA response occurs prior to, or during worm expulsion (Poulain *et al.*, 1976a). Ferguson and Jarrett (1975) have proposed that T-lymphocytes in the mucosa could be responsbie for the partial villous atrophy which occurs during infection. However, there has been no direct demonstration that mucosal lymphocytes have any protective function.

Although histological changes amongst intestinal epithelial cells in rats infected with *N. brasiliensis* have been reported by Symons and

co-workers (Symons, 1957; Symons and Fairbairn, 1963; Symons, 1965) there is, at present, no direct evidence that epithelial cells serve any protective purpose. However, goblet cells were found to be increased in number during infection with *N. brasiliensis* (Wells, 1963) and consideringthe reported parasiticidal activity of mucus in chickens (Frick and Ackert, 1948) it is possible that mucus could play a role in worm expulsion.

3.5 Hypersensitivity reactions involved in protective immunity

In the rat, anaphylactic shock is characterized by progressive lesions appearing in the small intestine (Sanyal and West, 1958; West, 1959). Urquhart *et al.* (1965) demonstrated increased vascular permeability in rats harbouring *N. brasiliensis* at the sites occupied by the adult worms. They also demonstrated an increase in intestinal mucosal permeability after the induction of anaphylaxis by intravenous injection of worm extracts in rats either infected with parasites or passively sensitized with immune sera. They concluded that local anaphylactic reactions in the intestine gave rise to conditions which were 'unsuitable' for the worms. This idea has been further expanded by Barth *et al.* (1966) who showed that an ovalbumin induced anaphylactic reaction could cause rats which had been passively immunized with anti-*N. brasiliensis* serum, to expel a transplanted adult worm population more quickly than the appropriate controls. These results, together with the morphological changes of the intestinal epithelium in the shocked rats, led to the proposal (Barth *et al.*, 1966) that the increased mucosal permeability associated with the local anaphylactic reaction might facilitate the passage or 'leakage' of significant amounts of antibody into the gut lumen. This has been called the 'leak lesion' hypothesis (Ogilvie and Jones, 1973). Additional support for this concept was the demonstration of a macromolecular leakage during *N. brasiliensis* infection (Halliday, Neilson and Mulligan, 1965; Neilson, 1969; Murray *et al.*, 1971a). Moreover, Murray

and co-workers (Murray *et al.*, 1971a; Murray, Jarrett, Jennings and Miller, 1969; Murray, Smith, Waddell and Jarrett, 1971c) demonstrated a correlation between the onset of worm expulsion, the macromolecular leak into the gut lumen and the intestinal mast cell responses. These results, together with reports on the appearance of reaginic antibodies at about the time of worm expulsion (Ogilvie, 1964, 1967; Ogilvie and Jones, 1969), suggested that the interaction of antigen with IgE on the mast cell surface might play an important role in the pathogenesis of the 'leak-lesion' (Rev. by Murray, 1972). Wilson and Bloch (1968), who described the early sensitization of peritoneal mast cells, supported the 'leak-lesion' hypothesis and, in addition, suggested that IgE might also be capable of sensitizing endothelial structures directly so as to alter capillary permeability on contact with antigen (Bloch, 1967).

The 'leak-lesion' hypothesis has been challenged by several workers, for example, Keller (1971) was unable to relate the intestinal histamine levels and the mast cell response in *N. brasiliensis* infected rats to worm expulsion. Similarly, Kelly and Ogilvie (1972) reported that worm expulsion preceded the intestinal mast cell response in normal rats and that a rise in mast cell numbers occurred in lactating rats even though they were unable to expel their worm burden. Jarrett, Urquhart and Douthwaite (1969) were also unable to relate worm expulsion to increased mast cell numbers in baby rats nor could Keller (1970a) confirm that peritoneal mast cells were sensitized around about the time of worm expulsion. Finally, Ogilvie and co-workers (1977) reported that transplanted 'damaged' worms were expelled from heavily irradiated (750 r) recipients by transfer of immune TDL without any accompanying intestinal mast cell or eosinophil response.

These findings appear to discredit the role of anaphylaxis in the protective mechanisms against the parasite, although some caution should be

used in the interpretation of the conflicting data until several of the parameters have been re-examined in detail. For example, measurement of mucosal permeability would be affected by a variety of factors such as mucosal haemodynamics, capillary permeability, epithelial permeability and worm burden and although all classes of plasma protein may leak into the intestinal lumen, their heterogeneity complicates the measurement of any such leakage (Jeffries, Halsted, Holman, Sleisenger, 1962). Similarly, the IMC response is the outcome of complex interactions between host and parasite. Thus mast cell responses can be stimulated by infection with *N. brasiliensis* but, at the same time, these worms release a mast cell degranulator (Miller, 1969). For this reason such cells may be difficult to identify in histological sections. Nor is it known what critical mass of mast cells is necessary to produce an effect on the mucosa and on worm expulsion.

Other mediators of anaphylaxis, such as catecholamines, acetylcholine, SRS-A and kinins may be involved in the protective mechanisms (Rev. by Boreham and Wright, 1976) although there has been no definitive work on such mediators. However, a series of studies done by Dineen and co-workers (Rev. by Kelly and Dineen, 1976) suggest that prostaglandin E is the mediator which damages worms and, thus, causes worm expulsion.

Treatment with drugs such as amine antagonists (Urquhart *et al.*, 1965; Murray, Smith, Waddell and Jarrett, 1971c; Keller and Ogilvie, 1972) and amine depletors (Sharp and Jarrett, 1968; Keller, 1970b; Keller and Ogilvie, 1972) has been used to probe the role of anaphylactic responses in the expulsion of *N. brasiliensis*. Although the results lend support to a role for such mechanisms of protection, it is important to evaluate the effects of these drugs in more detail because the mediators of anaphylaxis have not been clearly identified and because most drugs have side effects.

For example, although it has been widely accepted that the major mediator of anaphylaxis in rats is 5-HT (Rev. by Austen and Humphrey, 1963), West (1959) suggested that neither histamine nor 5-HT were involved in the onset of anaphylaxis. Furthermore, Riley (1959) found that treatment with compound 48/80 did not affect the intestinal histamine levels in the rat and this was confirmed by Johnson (1968). However, treatment with this drug does not cause any decrease in IMC even though connective tissue mast cells are destroyed (Enerbäck, 1966c).

Keller and Ogilvie (1972) have discussed the possible side effects of drug treatment and have emphasised the drawbacks of such an approach, moreover, Kelly and Dineen (1972b) demonstrated that serum from promethazine-treated rats suppressed the activity of adoptively transferred immune lymphocytes. Corticosteroids have also been used to inhibit worm expulsion with the aim of analysing the immune response (Weinstein, 1955; Urquhart *et al.*, 1965; Ogilvie, 1965b; Murray *et al.*, 1971a; Keller and Ogilvie, 1972) and although cortisone and its derivatives are potent inhibitors of worm expulsion, their effects are multivalent and could be acting at several different levels during the response.

Cell-mediated (DTH) responses, are also provoked by helminth infections (Rev. by Jarrett and Urquhart, 1971; Rev. by Ogilvie and Jones, 1973). Production of migration inhibitory factor (MIF) by lymphoid cells sensitized to *N. brasiliensis* has been reported (Malczewski, Zaleska-Rutczynsky, Skopinska and Ostrowski, 1970; Blundell-Hasell, 1974a, b, c). Delayed skin hypersensitivity reactions were also demonstrated in rats 2 or 3 weeks after infection with *N. brasiliensis* (Blundell-Hasell, 1974c) but whether DTH has any effect on worm expulsion remains unknown. As was pointed out earlier in this introduction protection against *N. brasiliensis* can be adoptively transferred by immune lymphoid cells, although significant protection is

also passively transferred by immune serum. This would suggest that DTH may not be a major component of the expulsive mechanism since the hallmark of cell-mediated immunity is that it can be transferred to non-immune recipients only with viable lymphoid cells.

SCOPE OF THESIS

The intestinal tract is armed with a wide range of specific and non-specific protective mechanisms directed against noxious stimuli from the outer environment. Yet, despite considerable knowledge concerning secretory antibody of the IgA class, the general understanding of mucosal defence mechanisms has been hampered by a lack of suitable laboratory models, especially where some measurement of the host's protective response is required. It is particularly important to determine how protection at the mucosal surface is achieved against organisms which do not penetrate the epithelium since this is an area of mucosal immunology which is least understood. *Nippostrongylus brasiliensis* infection in rats is a suitable model for studies of mucosal immunity because the adult worms parasitize the surface of the mucosa (Symons, 1976) and because the immune status of the host can readily be measured either by monitoring faecal egg counts or by counting the worm burden. However, it is clear from this review of the literature that the parasite is a complex and persistent antigen and that the immune response against it is a multi-component event.

Although manipulation of the response to *N. brasiliensis* by the passive transfer of immune serum or by the adoptive transfer of immune lymphocytes should, in theory, prove helpful for the understanding of the mechanisms of mucosal protection, the relative roles of antibody and of cells in the mucous membranes are not well understood. The effects of adoptive immunization have been particularly confusing mainly because the source, dose,

and nature of the transferred cells have not been systematically studied. Despite the observation that the adoptive transfer of immune lymphocytes causes the earlier expulsion of the parasites from the intestine, it is not known whether such cells exert a direct effect against the parasite or whether they mediate indirectly by setting into motion other protective mechanisms.

The main aim of the present work is, therefore, to examine some of the parameters which determine the efficacy with which different cell populations effect worm expulsion. To this end, different cell sources taken from donors of different immune status are compared for their ability to confer protection against the intestinal phase of the parasite. The adoptive response is further analysed by fractionating the effector cells and examining the protective activities of each subpopulation.

It is hoped that this approach, in conjunction with histological studies of the mucosal changes in normal and in adoptively immunized rats, will shed some light on the role of immune lymphocytes in mucosal immune responses. Intestinal mast cells, IgA-synthesizing cells, and serum IgE levels will be examined in normal, and in adoptively immunized, infected rats. These studies also raise a number of questions as to the origins and functions of intestinal mast cells and of IgA- and IgE-synthesizing cells. The hypothesis that intestinal mast cells, together with IgE antibodies, are instrumental in causing increased mucosal permeability, thus permitting the translocation of anti-worm antibodies into the gut lumen will be re-examined.

CHAPTER 2

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

Female rats 11-13 weeks old were used for all experiments. Outbred Wistars and the inbred DA and PvG/c strains and their hybrid (PvG/c × DA)F_1 were examined for strain differences in the response to the parasite. Wistar rats were also used for the maintenance of the parasites, for harvesting adult worms to prepare worm antigen, and for the assay of passive cutaneous anaphylaxis (PCA). (PvG/c × DA)F_1 rats were used both as donors and as recipients for cell transfer experiments. All the surgical procedures were carried out under ether anaesthesia.

All the rats were bred within the Animal Breeding Establishment of the John Curtin School of Medical Research and were fed a diet of Rat and Mouse Cubes (Bunge Pty. Ltd, Australia) and given tap water *ad libitum*.

Infected animals were kept in metabolic cages with wire mesh floors suspended above plastic trays containing tap water for the collection of faeces to monitor the daily faecal egg production.

BASIC BIOLOGICAL SOLUTIONS

Phosphate buffered saline (PBS)

This is a 0.02M phosphate buffer pH 7.4 containing 0.15M NaCl and is prepared from the following stock solutions.

Stock solution I : 1.5M NaCl ... 87.66g NaCl was dissolved in 1 litre of distilled water

Stock solution II : 1M phosphate buffer pH 7.4 ... 500 ml of 1M K_2HPO_4 (228.23g $K_2HPO_4.3H_20$ in 1 litre of distilled water) and approximately 135 ml of 1M KH_2PO_4 (136.09 g KH_2PO_4 in 1 litre of distilled water) were mixed and adjusted to pH 7.4

One litre of stock solution I and 200 ml of stock solution II were mixed

and were made up to 10 litres with distilled water.

Tris buffered saline (TBS)

A 10 times concentrated stock solution was prepared by dissolving 30.3 g Tris (hydroxymethyl) aminomethane (Sigma 7-9), 81.8 g NaCl and 1 g NaN_3 in about 800 ml of distilled water and, after the pH was adjusted to 7.5 with 6N HCl, was made up to 1 litre with distilled water. When this solution was diluted 10 times before use, the pH stabilised at 7.4

Hank's balanced salt solution containing 1% bovine serum albumin (Hank's BSA)

This was prepared by dissolving the following chemicals in 1 litre of distilled water: 8.0 g NaCl, 0.4 g KCl, 0.1 g Mg $SO_4.7H_2O$, 0.14 g Mg $Cl_2.6H_2O$, 0.069 g Na_2HPO_4, 0.069 g KH_2PO_4 and 1.0 g glucose. Five ml of a 4% aqueous solution of phenol red was added to each litre as a pH indicator. Sterilized Hank's solution was supplied by the Department of Microbiology, John Curtin School of Medical Research. One per cent bovine serum albumin (Armour, England) was added to the solution before the pH was adjusted to 7.4 with 5% $NaHCO_3$. Hank's BSA was sterilized by passage through sterile 0.45 µ Millipore filters (HAWP 02500, Millipore Co).

Medium 199

Ten times concentrated cell culture medium 199 (Commonwealth Serum Laboratories, Melbourne) was diluted with sterile distilled water and the pH was adjusted to 7.4 with 5% $NaHCO_3$. Twenty mg of heparin (Pularin, Evans Medical Ltd. Liverpool, 151 units of heparin/mg) was added to 133.3 ml of medium 199 before adjusting the pH.

FIXATIVES

Carnoy's fluid

One hundred ml of this fixative was made up with 30 ml of chloroform, 60 ml of absolute ethanol and 10 ml of glacial acetic acid.

Cold ethanol (Sainte-Marie, 1962)

Ninety-five per cent ethanol was kept at 4°C.

4% paraformaldehyde-1% glutaraldehyde (Miller and Adams, 1977)

Ten grammes of paraformaldehyde were dissolved in 200 ml of PBS by heating and stirring on a hot plate. After cooling the solution to room temperature, 10 ml of 25% glutaraldehyde solution (TAAB) was added and the final volume was adjusted to 250 ml with PBS.

2% paraformaldehyde

Five grammes of paraformaldehyde were dissolved in 250 ml of PBS in the manner described above and the fixative was cooled to room temperature before use.

Glutaraldehyde-paraformaldehyde fixative for electron microscopy

To make up 70 ml of this solution,1.4 g of paraformaldehyde was dissolved by heating and stirring in 35 ml of 0.2M cacodylate buffer pH 7.4. After cooling to room temperature, 2 ml of 70% glutaraldehyde (LADD) was added to the solution and the volume was adjusted to 70 ml with distilled water. Finally, 0.15 ml of 1M $CaCl_2$ was added to the mixture. This fixative contains 2% glutaraldehyde, 2% formaldehyde and 0.026% $CaCl_2$ in 0.1M cacodylate buffer pH 7.4.

PARASITOLOGICAL TECHNIQUES

The parasite

The strain of *N. brasiliensis* used in these experiments was originally established and maintained in the McMaster Laboratory, CSIRO, Sydney, and is now maintained in our laboratory by passage in outbred Wistar rats.

Faecal culture and the preparation of infective larvae

Faeces from rats infected with 4000 L_3 were collected in plastic trays containing tap water between days 6 and 9 of infection. They were washed in tap water to remove urine and the cultures were set up using a slight

modification of the method described by Jennings, Mulligan and Urquhart (1963). Briefly, the faeces were homogenised and smeared on 5.5 cm diameter Whatman No. 1 filter papers which were placed on discs of synthetic sponge 1/2 cm thick and 3 cm diameter. These were placed on 9.0 cm Whatman No. 1 filter papers in 9 cm diameter petri dishes containing 2-5 ml distilled water. The cultures were kept in a moist 26°C incubator. With this method, the larvae migrate to the edge and the under-surface of the bottom filter paper. After 5 days of incubation the faecal smears and the sponges were removed and PBS was added to the petri dish to suspend the larvae. The bottom filter paper was rinsed and removed and the larval suspensions were harvested and were washed several times with PBS by sedimentation and decantation. The larvae were allowed to migrate through a 200 steel mesh at 37°C for 10 min. in PBS to remove dead larvae and fragments of filter paper. Larval suspensions were adjusted to the required concentration and doses of 0.5 ml were inoculated subcutaneously into the flank region.

Faecal egg counts

Faecal egg counts were monitored using a modification of the McMaster slide counting technique described by Whitlock (1948).

Method for counting worm burdens

The whole length of the small intestine was opened longitudinally, was enveloped in surgical gauze, and was suspended in pre-heated (37°C) PBS and incubated at 37°C for 1 hour. Although the majority of worms migrate out through the gauze with this technique, it was found that 5-15% remained trapped in the intestine and the gauze. These residual parasites were counted by examining the intestine and its contents, as well as the gauze, under a dissecting microscope.

Anthelmintic treatment

Anthelmintic treatment was carried out according to the method described by Murray *et al*, (1971a). Animals were given 220 mg/kg body weight of thiabendazole (Thibenzole, Merck, Sharp and Dohme) by stomach tube.

Intraduodenal implantation of adult worms

Adult worms were obtained from the intestines of (PvG/c × DA)F_1 rats 6 days ('normal' worms) or 11 days ('damaged' worms) after primary infection with 4000 L_3. The worms were washed several times with PBS, they were counted and suspended in 1 ml of sterile saline before they were introduced surgically into the proximal duodenum of recipient (PvG/c × DA)F_1 rats with a 1 ml syringe fitted with an 18 G hypodermic needle. Six samples of worms were selected at random and recounted in order to check the error.

PURIFICATION OF RAT IMMUNOGLOBULINS

Measurement of protein concentrations

The protein concentration of the purified immunoglobulins and their fragments was determined by measuring the optical density at 280 nm with a Hitachi spectrophotometer model 101 assuming an average $E^{1\%}_{1\ cm} = 13$ for this class of protein and for its fragments.

Immunoelectrophoresis and double immunodiffusion

The buffer solution was prepared by dissolving 24.5 g of veronal buffer salts (LKB 3276-VB, Sweden) in 1500 ml of distilled water. The pH of this solution was 8.6 and its ionic strength, $\mu = 0.1$.

One gram of agar (Difco special agar noble) was dissolved in 100 ml of veronal buffer and 3 ml of this 1% agar solution was poured onto glass microscope slides (3" × 1"). Wells were punched in the gel using a gel-puncher (LKB Type 6808A) for immunoelectrophoresis. Two micro-litre samples were put in the wells by means of a Hamilton syringe and were electrophoresed for 90 min. at 200 V constant voltage using a LKB Type 3290B immunoelectrophoresis apparatus. After the completion of electrophoresis, a central

trough was cut in each gel and 75 µl of antiserum was placed in the trough. The precipitin lines were allowed to develop overnight (15-18 hours). The gel plates were washed twice with PBS overnight then twice with distilled water overnight. After drying the gel at room temperature, the precipitin lines were stained with Amido-black 10B or Ponceau S and washed with 10% acetic acid-50% methanol.

For double immunodiffusion, wells were punched at 5 mm distance using an LKB template and gel puncher in gels poured onto microscope slides as described for electrophoresis. Ten microlitres of the antigens, of the antisera, or of the purified antibodies were placed into each well using a Finnpipette (Ky Finnpipette). To test the purity of antigens (or antibodies), six wells were punched concentrically at a distance of 5 mm from a central well. Ten microlitres of doubling dilutions of the samples (original concentration: 2-5 mg protein/ml) were placed in the surrounding wells and 10 µl of antibody (or antigens: 2-5 mg protein/ml),were loaded into the central well. The procedures for developing precipitin lines and for washing and staining the gels were the same as those described above for immunoelectrophoresis.

Sepharose 4B immunosorbants

The method of coupling proteins to Sepharose 4B was that described by Gerber (1976). Briefly, 4.2 g anhydrous Na_2CO_3 was dissolved in 20 ml of a 50% suspension of Sepharose 4B (Pharmacia). This alkaline suspension was then immediately added to a stirred 10% solution of cyanogen bromide. The suspension was stirred for a further 2 min. before the activated gel was washed 3 times with ice-cold distilled water on a sintered glass filter (G_2). The CNBr-activated Sepharose beads were collected and immediately transferred into 10 ml of protein solution (up to 200 mg protein) previously dialyzed against 0.2M borate buffer pH 9.5 containing 0.5M NaCl. The suspension was gently stirred for at least 3 hours at room temperature.

After measuring the uncoupled protein, the immunosorbant was washed extensively with TBS to block free active binding sites, then washed once with 0.2M glycine-HCl buffer pH 2.2 and finally with TBS.

Purification of normal rat IgG_{2a}

The methods used were essentially those described by Bazin *et al.* (1974). A crude globulin fraction was precipitated from pooled normal Wistar serum with 50% saturated ammonium sulphate and was dissolved and dialysed against 0.05M Tris-HCl buffer pH 8.0. After removal of a small amount of precipitate by centrifugation, 300 mg of protein was applied to a DEAE-Sephadex A50 (Pharmacia) column (1.6 × 25 cm) equilibrated with 0.05M Tris-HCl buffer pH 8.0. The ascending portion of the first peak eluted with the same buffer but with the addition of 0.05M NaCl, was concentrated, dialysed, and rechromatographed in the manner described above. The ascending portion of the first peak was, after concentration, identified as pure IgG_{2a} by immuno-electrophoresis (Fig. 2-1) and double diffusion against rabbit anti-whole rat serum (see production of antisera in this chapter).

Fab and $F(ab')_2$ fragments from IgG_{2a}

Papain digestion was carried out at a protein:enzyme ratio of 100:2 in 0.1M phosphate buffer pH 7.5 containing 0.002M EDTA and 0.01M L-cysteine at 37°C for 18 hours (Porter, 1959). To stop the reaction 1/20 volume of 0.4M iodoacetamide was added to the digest (Marler, Nelson and Tanford, 1964). After dialysis against 0.01M phosphate buffer pH 8.1 containing 0.15M NaCl, the digested material was filtered through a 1.6 × 70 cm column of Sephadex G100 (Pharmacia). The major peak was pooled and concentrated, and then dialysed against 0.01M phosphate buffer pH 7.1. This material was chromatographed on a DEAE cellulose (DE52, Whatman) column (0.8 × 15 cm) equilibrated with 0.01M phosphate buffer pH 7.1 (Armerding, 1971). The

protein eluted with same buffer was concentrated to 5 mg/ml and dialysed against TBS. This was identified as pure Fab by immunoelectrophoresis and by double immunodiffusion.

To obtain $F(ab')_2$ fragments, pepsin was added to purified normal rat IgG_{2a} (10 mg/ml) previously dialysed against 0.07M acetate buffer pH 4.0 containing 0.05M NaCl at a protein:enzyme ratio of 100:3 (Nisonoff, Wissler, Lipman and Woernley, 1960). A drop of toluene was added to prevent bacterial growth. Incubation was carried out at 37°C for 18 hours and the reaction was stopped by adding solid tris which neutralized the mixture. After neutralization, the digested material was dialysed against TBS and concentrated, and was then applied to a Sephadex G200 column (2.6 × 70 cm). The central portion of the major peak which was eluted in a region between the 7S and 4S peaks was pooled and concentrated, and was identified as pure $F(ab')_2$ by immunoelectrophoresis and by double immunodiffusion.

Purification of rat IgA

Rat IgA in serum and secretions was first identified by Nash, *et al.* (1969). The occurrence of relatively high concentrations of IgA in the thoracic duct lymph of rats has been reported by Nash and Heremans (1972). Because it is readily obtained in large volumes, thoracic duct lymph would appear to be a suitable source from which to purify IgA when compared to other sources such as colostrum (Stechschulte and Austen, 1970) and saliva (Bistany and Tomasi, 1970).

Thoracic duct lymph collected from normal $(PvG/c \times DA)F_1$ rats was centrifuged to remove cells and was concentrated to 1/10 of its original volume by ultrafiltration *in vacuo*. Chylomicrons and lipoproteins were precipitated with high molecular weight dextran sulphate in the presence of calcium and were removed by centrifugation (Walton and Scott, 1964). A clear supernatant was obtained and this served as the starting material

Fig. 2-1: Immunoelectrophoretic pattern of purified rat IgG_2a. Two µl of purified rat IgG_2a (5 mg/ml) (top well) and 2 µl of pooled normal rat serum (bottom well) were electrophoresed and were developed with 75 µl of rabbit antiserum against rat whole serum. The gels were dried and stained with Ponceau S.

Fig. 2-2: Specificity of sheep anti-rat IgA antiserum.

(a) Immunoelectrophoresis: Two µl of pooled normal rat serum (top well) and 2 µl of 10 times concentrated and defatted normal rat thoracic duct lymph (bottom well) were electrophoresed and developed with 75 µl of sheep anti-rat IgA antiserum which was previously absorbed with an SPF rat serum immunosorbent. Stained with Ponceau S.

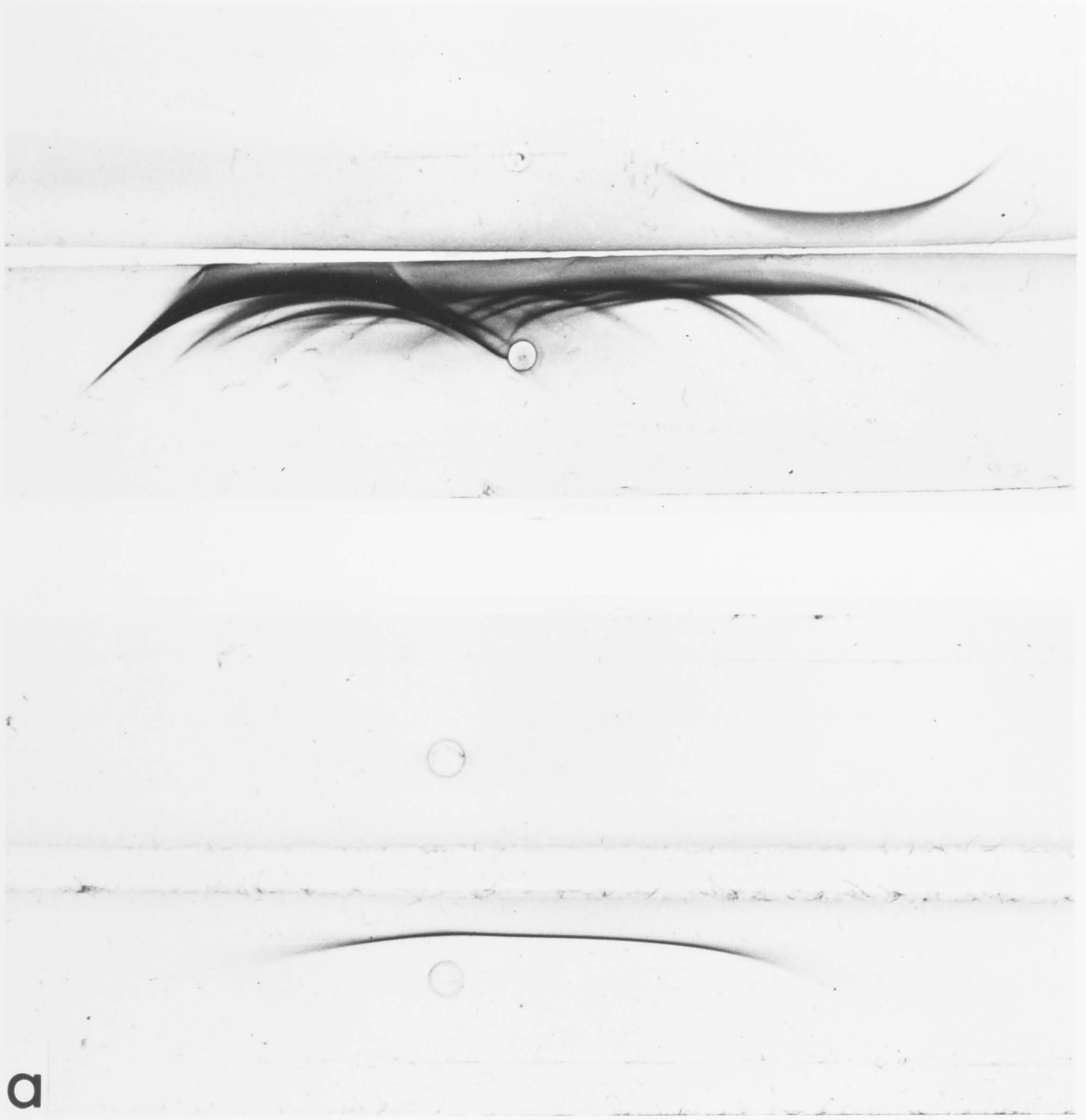
(b) Double immunodiffusion: Ten µl of the following samples were applied to each well:

Top left: normal rat thoracic duct lymph (described above)

Top right: pooled normal rat serum

Bottom left and right: sheep anti-rat IgA antiserum (described above)

Bottom middle: goat anti-rat IgA antiserum provided by Dr. H. Bazin.



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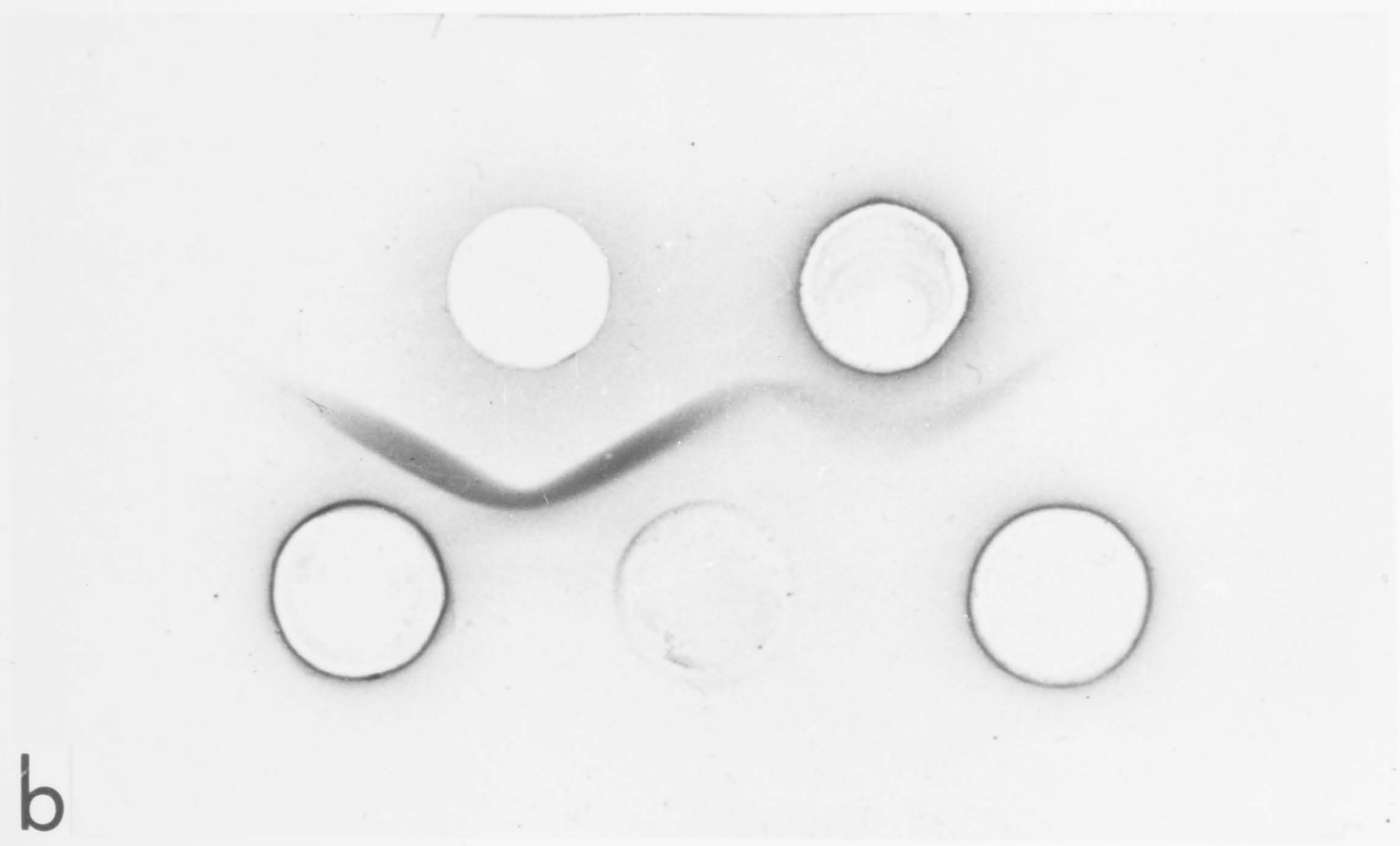


b

for the purification of IgA. The preparative techniques used were essentially those described by Heremans (1965). Briefly, the starting material was dialysed against 0.02M phosphate buffer pH 6.5 and euglobulin precipitates were removed by centrifugation and discarded. A crude immunoglobulin fraction, salted out from the supernatant with 2M ammonium sulphate, was dissolved and dialysed against McIlvain's phosphate-citrate buffer pH 5.0, and was then applied to a CM-cellulose (CM52, Whatman) column (3 × 20 cm) equilibrated with the same buffer. The proteins eluted with this buffer were pooled and were concentrated by pressure dialysis using an ultrafiltration apparatus fitted with a PM-10 membrane (Amicon). Immunoelectrophoresis revealed that at this stage the protein solution contained alpha-globulins and albumin in addition to IgA but there was no detectable IgG or IgM. After dialysis against TBS, this material was applied to a sheep Ig anti-rat Fab immunosorbant column. The protein eluted with 0.2M glycine-HCl buffer pH 2.2 was neutralised and dialysed against TBS. After concentration, it appeared to be pure IgA by immunoelectrophoresis and by double immunodiffusion.

PRODUCTION OF ANTISERA

Rabbit anti-whole rat serum

One millilitre samples of pooled normal Wistar rat serum were emulsified in equal volumes of Freund's complete adjuvant (Difco) and were injected intramuscularly into the hind legs of several rabbits. The rabbits were boosted 1 month later by the same procedure and were bled from the ear vein 1 week after boosting. Further boosting and bleeding were performed 2 and 4 months later.

Sheep anti-rat Fab

A 2.5 ml saline solution containing 1 mg of purified rat Fab was emulsified in 2.5 ml of Freund's complete adjuvant and was injected

subcutaneously bilaterally into the tarsal region as well as intramuscularly into the gluteal region of a sheep. A booster injection was given 1 month later and the sheep was bled 1 week after boosting.

Sheep and rabbit anti-rat IgA

A sheep and 2 rabbits were immunized 3 times at weekly intervals with 0.5 mg of a purified IgA fraction (see above) incorporated into Freund's complete adjuvant. The animals were bled 1 week after the final injection and the antisera were found to have, in addition to anti-IgA activity, anti-Fab activity and anti-alpha globulin activity. The contaminating antibodies were removed after passing the sera through an immunosorbant of specific pathogen-free rat serum coupled to Sepharose 4B. The specificity of these anti-rat IgA sera after absorption was confirmed by immunoelectrophoresis and by double diffusion where it was compared with goat and rabbit anti-rat IgA kindly provided by Dr. H. Bazin (Figs. 2-2a, b). The specificity of this and of Dr. Bazin's anti-IgA antisera was also checked at the cellular level (see this chapter, immunoperoxidase techniques).

Rabbit anti-sheep $F(ab')_2$

A solution containing 1 mg of sheep $F(ab')_2$ fragments prepared from sheep anti-rat Fab antiserum (see the following section in this chapter, 'purification of antibodies and their fragments') was emulsified in Freund's complete adjuvant and injected into rabbits 3 times at weekly intervals. The rabbits were bled 1 week after the last challenge and, following a further booster injection of 1 mg sheep $F(ab')_2$ incorporated in Freund's incomplete adjuvant, bleeding was repeated several times at 1 month intervals in order to obtain sufficient serum.

Sheep anti-rabbit $F(ab')_2$

Rabbit $F(ab')_2$ was prepared and supplied by Dr. H.R.P. Miller (Immunology, JCSMR). One mg of rabbit $F(ab')_2$ was incorporated in Freund's

complete adjuvant and was injected into sheep in the manner described for the preparation of sheep anti-rat Fab. The sheep was bled 1 week after boosting.

PURIFICATION OF SPECIFIC ANTIBODIES AND THEIR FRAGMENTS

Sheep anti-rat Fab antiserum was applied to a rat $F(ab')_2$ immunosorbant column and specific sheep Ig anti-rat Fab was eluted with 0.2M glycine-HCl buffer pH 2.2. The eluate was neutralised with solid tris and was dialyzed against TBS before it was purified by gel filtration through Sephadex G200 to remove aggregates.

To obtain specific $F(ab')_2$ antibodies, a crude globulin fraction of the antiserum was precipitated with 50% saturated ammonium sulphate and the precipitate was dissolved and dialysed against 0.07M acetate buffer pH 4.0 containing 0.05M NaCl. Digestion with pepsin was as described previously and, after neutralisation, the digested material was applied to the appropriate Sepharose 4B immunosorbant column. The protein eluted with a glycine-HCl buffer was neutralised and was purified by gel filtration through Sephadex G200. The purity of these $F(ab')_2$ preparations was checked by double diffusion against monospecific antisera.

Sheep Ig anti-rabbit $F(ab')_2$ and rabbit $F(ab')_2$ anti-sheep $F(ab')_2$ were purified using rabbit $F(ab')_2$ and sheep $(Fab')_2$ immunosorbant columns respectively.

IMMUNOPEROXIDASE TECHNIQUES

Conjugation

Horseradish peroxidase (HPO: Type VI Sigma) was coupled to sheep Ig anti-rat Fab, to sheep $F(ab')_2$ anti-rat Fab, to sheep Ig anti-rabbit $F(ab')_2$ and to rabbit $F(ab')_2$ anti-sheep $F(ab')_2$ using the method described by Nakane and Kawaoi (1974). Briefly, 5 mg of HPO was dissolved in 1.0 ml of freshly made 0.3M bicarbonate buffer pH 8.1 and 0.1 ml of 1% fluorodinitrobenzene

(FDNB) in absolute ethanol was added and was mixed gently for 1 hour at room temperature. One ml of 0.05M $NaIO_4$ in distilled water was added to the above mixture which was gently stirred for 30 min. at room temperature. The reaction was stopped by the addition of 0.1 ml of 1M ethylene glycol in distilled water. After 1 hour of gentle mixing, the solution was dialysed against three changes of 0.01M carbonate buffer pH 9.5 at 4°C. Three mg of activated HPO was added to 7.5-10 mg Ig or to 5 mg $F(ab')_2$ fragments previously dialysed against 0.01M carbonate buffer pH 9.5. Conjugation was carried out for 2-3 hours with gentle stirring at room temperature before 5 mg of $NaBH_4$ was added to stop the reaction. After mixing the $NaBH_4$ for 30 min. at room temperature, one drop of acetone was added to break down $NaBH_4$. Conjugates were purified by gel filtration through Sephadex G200 to remove uncoupled materials. A typical elution pattern of sheep Ig anti-rat Fab conjugated with HPO is shown in Fig. 2-3.

Immunoperoxidase staining of surface and intracellular Ig

The methods were essentially according to those described by Miller and Adams (1977). To stain surface Ig directly, 5×10^6 washed TDL were incubated with 30 µl of sheep $F(ab')_2$ anti-rat Fab-HPO (1 mg/ml) and 30 µl of Hank's-BSA for 1 hour at 4°C. Preliminary titrations had shown that this concentration of conjugate was optimal for surface labelling. For indirect labelling, the same number of cells was incubated with 30 µl of unlabelled sheep $F(ab')_2$ anti-rat Fab (100 µg/ml) and 30 µl of Hank's-BSA for 30 min. at 4°C. The cells were washed three times with cold Hank's-BSA and were incubated with 30 µl of rabbit $F(ab')_2$ anti-sheep $F(ab')_2$-HPO (1 mg/ml) and 30 µl of Hank's BSA for 30 min at 4°C. After the final incubation the cells were washed 3 times with cold Hank's-BSA and were smeared by cyto-centrifugation. Smears were fixed in 4% paraformaldehyde-1%

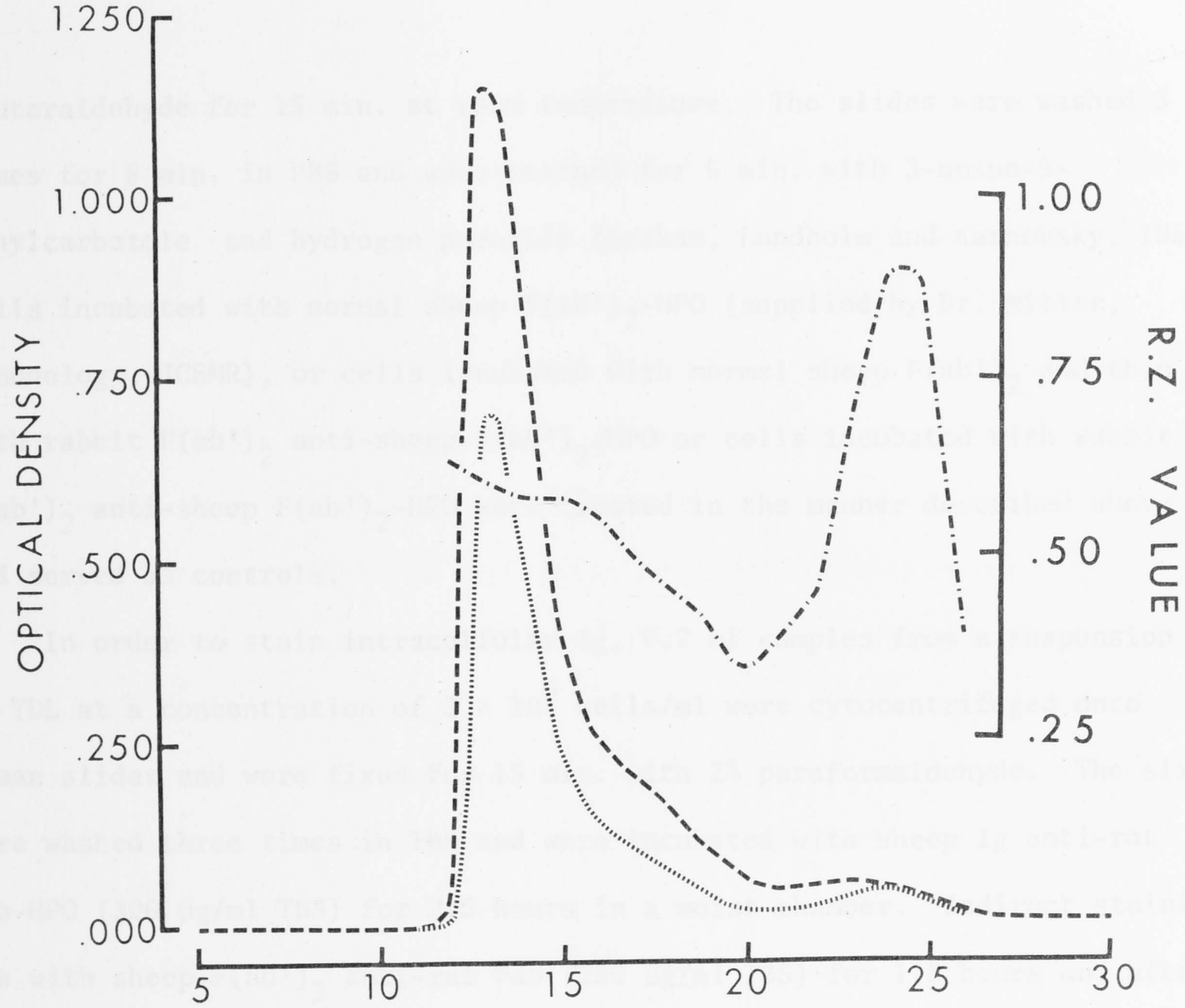


Fig. 2-3: Typical elution pattern of 10 mg of sheep Ig anti-rat Fab conjugated with 3 mg of activated HPO. Ascending chromatography was carried out on a 1.6 × 70 cm column of Sephadex G200. Flow rate was 9 ml/hour and the eluting buffer was TBS. Optical densities were measured at 280 nm (-----) and 403 nm (.....). R.Z. ratios of absorbance at 403 nm to absorbance at 280 nm of each fraction were calculated and were also plotted on this graph (- · - · - · -).

glutaraldehyde for 15 min. at room temperature. The slides were washed 3 times for 5 min. in PBS and were stained for 5 min. with 3-amino-9-ethylcarbazole and hydrogen peroxide (Graham, Lundholm and Karnovsky, 1965). Cells incubated with normal sheep $F(ab')_2$-HPO (supplied by Dr. Miller, Immunology, JCSMR), or cells incubated with normal sheep $F(ab')_2$ and then with rabbit $F(ab')_2$ anti-sheep $F(ab')_2$-HPO or cells incubated with rabbit $F(ab')_2$ anti-sheep $F(ab')_2$-HPO were treated in the manner described above and served as controls.

In order to stain intracellular Ig, 0.2 ml samples from a suspension of TDL at a concentration of 2×10^6 cells/ml were cytocentrifuged onto clean slides and were fixed for 15 min. with 2% paraformaldehyde. The slides were washed three times in TBS and were incubated with sheep Ig anti-rat Fab-HPO (300 µg/ml TBS) for 2.5 hours in a moist chamber. Indirect staining was with sheep $F(ab')_2$ anti-rat Fab (250 µg/ml TBS) for 1.5 hours and after washing 3 times with TBS the slides were incubated with rabbit $F(ab')_2$ anti-sheep $F(ab')_2$-HPO for 1 hour in a moist chamber at room temperature. Slides incubated with normal sheep Ig-HPO, with normal sheep $F(ab')_2$ and then with rabbit $F(ab')_2$ anti-sheep $F(ab')_2$-HPO or with rabbit $F(ab')_2$ anti-sheep $F(ab')_2$-HPO alone served as controls. After the final incubation the slides were washed 3 times for 5 min. in TBS and were stained by the carbazole technique described above.

IgA-synthesising cells in the intestinal lamina propria

Approximately 1.5 cm of jejunum was removed, unopened, at a point about 20 cm behind the pylorus. The excised piece of intestine was laid on a card, opened along its mesenteric attachment and, after counting the adherent parasites, the flattened segment was immersed in 95% ethanol at 4°C. Fixation and processing of the tissues were carried out according to the

method described by Sainte-Marie (1962). Sections were deparaffinised in xylene and were rehydrated in a descending ethanol series; they were then washed 3 times with TBS. Immersion in 0.1M periodic acid for 5 min. then in 0.02% sodium borohydride for 2 min. was used to inhibit endogenous peroxidase activity (Heyderman and Neville, 1977). The sections were washed with TBS and were incubated for 90 min. at room temperature in a moist chamber with rabbit anti-rat IgA serum (a gift from Dr. H. Bazin) which was diluted 20 times with TBS containing 10% normal rabbit serum. The slides were washed 3 times with TBS and then incubated with sheep Ig anti-rabbit $F(ab')_2$-HPO (50 µg/ml in TBS containing 10% normal sheep serum) for 60 min. Control sections were incubated with normal rabbit serum and then with sheep Ig anti-rabbit $F(ab')_2$ -HPO or, alternatively, with rabbit anti-rat IgA and then with normal sheep Ig-HPO or with normal sheep Ig-HPO alone. After incubation, the slides were washed 3 times with TBS and were stained with diaminobenzidine and hydrogen peroxide for 10 min. at room temperature (Graham and Karnovsky, 1966). Stained sections were washed with distilled water, dehydrated in an ascending ethanol series, cleared in xylene, and the coverslips were mounted with Histoclad (Clay Adams).

The specificity of the anti-rat IgA sera was also tested at the cellular level. Popliteal lymph nodes were taken from rats immunized 6 weeks previously in the footpads with 0.025 mg of ovalbumin emulsified in 0.05 ml of Freund's complete adjuvant. Cell smears were prepared by cytocentrifugation and indirect staining was performed according to the methods described above. Dilutions of 1/10-1/100 of the anti-rat IgA sera were tested. Some smears were incubated with sheep $F(ab')_2$ anti-rat Fab as positive controls. The smears were washed and then incubated with the appropriate reagents labelled with peroxidase, before being stained with carbazole. Less than 0.1% of cells were stained after incubation with anti-IgA sera whereas approximately 20% of cells stained for immunoglobulin

after incubation with anti-Fab.

The staining properties of the sheep anti-rat IgA antibody, the preparation of which is described in this chapter, were identical to those of the rabbit anti-IgA antiserum provided by Dr. H. Bazin. This specific antibody preparation was used to detect IgA-synthesizing cells in TDL whereas the rabbit anti-IgA serum provided by Dr. Bazin was used to detect mucosal IgA-synthesizing cells.

HISTOLOGICAL METHODS FOR THE DETECTION OF INTESTINAL MAST CELLS

Histological specimens were obtained according to the methods described previously in this chapter (see Intestinal IgA-synthesizing cells) except that the flattened jejunal segments were fixed in Carnoy's fluid. The fixed tissues were trimmed, further dehydrated in 3 changes of absolute ethanol and were cleared in chloroform before they were embedded in paraffin wax. Sections were stained with 0.5% Alcian blue at pH 0.3 for 30 min. and were rinsed in 0.7N HCl for 10 min. before they were counter-stained with 0.5% Safranin O at pH 1 for 30 seconds (Enerbäck, 1966a; Murray *et al.*, 1968). The method of counting intestinal mast cells was that described by Miller and Jarrett (1971). The number of mast cells lying between two gland crypts and in the lamina propria of the villus above was counted (villus-crypt unit: VCU). At least 30 VCU were counted in each section. Since globule leukocytes have already been identified as mast cells which are discharging or have discharged their granules (Murray *et al.*, 1968; Miller and Walshaw, 1972), they were included in the mast cell counts.

ELECTRON MICROSCOPY

Small segments (1.5 cm) of jejunum adjacent to those examined for mast cells and IgA-synthesizing cells were ligated at both ends and 0.05-0.1 ml of 2% glutaraldehyde-2% formaldehyde fixative were injected intraluminally. The segments were immediately removed and immersed in the same fixative and left overnight at 4°C. They were opened along their mesenteric attachments and, after counting the adherent parasites, each segment was trimmed along

its longitudinal axis and was washed three times with 0.1 M cacodylate buffer pH 7.4. Trimmed pieces were mounted in 7% agar and longitudinal thick sections (60-80 μ) were cut with a Smith-Farquhar tissue sectioner (Sorvall). The thick sections were washed once with cacodylate buffer and were incubated with diaminobenzidine in 0.1M Tris-HCl buffer pH 7.4 containing hydrogen peroxide for 30 min. (Graham and Karnovsky, 1966). They were then washed twice with cacodylate buffer, and post fixed in 2% osmium in 0.1M cacodylate buffer pH 7.4 for 30 min. at room temperature. After post fixation, the sections were washed twice with buffer and were dehydrated in an ascending acetone series and flat embedded in araldite (Durcupan, ACM, Fluka) so that the mucosa was oriented in the same way as the histological preparations.

One micron sections cut with glass knives were mounted on slides and were then stained with Azure 2-methylene blue-borax (Richardson, Jarrett and Finke, 1960). Thin sections cut with diamond knives were mounted on 200 mesh copper grids and stained with aqueous uranyl acetate and lead citrate. They were examined in a Philips EM300 electron microscope.

CELL TRANSFER

Primary infection cell donors were infected with 4000 L_3. Hyperimmune donors were infected 3-4 times with 4000 L_3 over a 1-2 month period.

Thoracic duct cannulation was carried out according to Gowans' (1959) modification of the Bollman technique (Bollman, Cain and Grindlay, 1948). Five units of heparin in 1 ml saline were injected subcutaneously into cell donors just after cannualtion. To increase lymph flow, 0.45% NaCl was given as drinking water. Thoracic duct lymph was collected overnight in sterile flasks containing 10 ml of Medium 199 and 20 units of heparin. Thoracic duct lymphocytes were harvested and washed 3 times in Hank's BSA and were finally adjusted to the required concentration of viable cells with Hank's-BSA. The viability of the cells was more than 95% as judged by phase-contrast microscopy.

Mesenteric lymph nodes were minced and gently squashed between the frosted ends of sterile microscope slides in Hank's BSA. The cell suspension was passed through two layers of surgical gauze to remove debris and was then prepared in the same way as the thoracic duct lymphocytes. The viability of MLNC was more than 85%. Recipient animals were injected with 1 ml of cell suspension via the lateral tail vein. Control rats were injected with 1 ml of Hank's BSA.

CELL FRACTIONATION

Fractionation of thoracic duct lymphocytes into subpopulations of cells bearing surface Ig (sIg^+ cells) and of cells lacking surface Ig (sIg^- cells) by a rosetting procedure was carried out in collaboration with Dr. C.R. Parish (Microbiology, JCSMR). The principles of the procedure and its application have been described previously by Parish and co-workers (Parish and Hayward, 1974a, b, c; Parish, Kirov, Bowern and Blanden, 1974).

Reagents

Antibodies Sheep $F(ab')_2$ anti-rat Fab and rabbit $F(ab')_2$ anti-sheep $F(ab')_2$ purified by immunoabsorption were dialysed against normal saline and were adjusted to 2 mg protein/ml in normal saline.

Separating medium This medium consisted of 12 parts of 14% (w/v) Ficoll (Pharmacia) dissolved in distilled water and 5 parts of 32.8% (w/v) sodium metrizoate (Isopaque; Nyegaard and Co.), the complete mixture containing 0.1% (w/v) sodium azide had a density of 1.09. It was sterilised by millipore filtration and was stored at 4°C protected from light.

Rosetting procedure

Sheep red blood cells (SRBC) were washed 4 times with normal saline and 0.25 ml of packed SRBC were suspended in 4 ml of normal saline. One hundred microlitres of rabbit $F(ab')_2$ anti-sheep $F(ab')_2$ (2 mg/ml in normal saline) and 150 µl of 0.1% $CrCl_3$ were added to the SRBC suspension and the

mixture was incubated for 10 min. at room temperature. The amount of $CrCl_3$ solution to be added was determined by a preliminary titration. The antibody-coated SRBC were washed twice with 2 ml of PBS and were finally suspended in 12.5 ml of PBS containing 10% foetal calf serum to make a 2% suspension.

Rat TDL were washed three times with Hank's-BSA and were adjusted to 4×10^7 cells/ml. Twenty microlitres of sheep $F(ab')_2$ anti-rat Fab (2mg/ml in normal saline) was added to 2.5 ml of the cell suspension (4×10^7 cells/ml; 1×10^8 total cells) which was incubated at 4°C for 30 min. The amount of sheep $F(ab')_2$ anti-rat Fab was determined by a series of preliminary titrations. The cells were centrifuged and were resuspended in 2.5 ml of Hank's-BSA before 2.5 ml of 2% antibody-coated SRBC were added to the cell suspension. The erythrocyte-lymphocyte mixture was incubated at 4°C for 30 min and was then centrifuged for 5 min. at 1200 Xg at 4°C to enhance rosette formation. The cell pellets were then gently resuspended and samples were taken and were diluted and examined in order to count rosettes.

Twenty microlitres of 25% (w/v) sodium azide (final concentration 0.1%) were added to 5.0 ml of the rosetted cell suspension and the mixture was brought to 20°C in a water bath. The suspension was then layered gently onto 4.0 ml of prewarmed (20°C) separating medium in 12 ml, U-bottomed, polycarbonate centrifuge tubes (16 × 100 mm, No. 272, Ivan Sorvall Inc.). After loading the cell suspension, the tubes were placed in a centrifuge prewarmed to 20°C and were centrifuged at 2000 Xg for 20 min. at 20°C.

After centrifugation, the supernatant above the interface was discarded and the white cell layer at the interface, together with all the separating medium above the red cell pellet, was collected. The white cell preparation ('upper layer') was then diluted with 10 ml of cold Hank's-BSA and the cells were centrifuged at 300 Xg for 10 min. at 4°C. The cells

were washed twice more with Hank's-BSA and were adjusted to the required concentration.

The pelleted red cells were lysed by osmotic shock. Each pellet was rapidly suspended in 8.55 ml of 40% Ringer's solution and, after all cell clumps had been dispersed, isotonicity was restored by the addition of 1.0 ml of 940% Ringer's solution. The white cells harvested by centrifugation at 300 Xg for 5 min. at 4°C were washed once with Hank's-BSA and cell numbers were counted and were adjusted to the required concentration.

INTESTINAL PERMEABILITY

To measure plasma protein translocation across the intestinal mucosa, Evan's blue was used as an indicator. Half a millilitre of 1% Evan's blue in saline was injected intravenously into each animal. The rats were exsanguinated under ether anaesthesia and the entire small and large intestines were removed and their outer surfaces were thoroughly wiped with damp surgical gauze to avoid contamination of the gut contents with blood. The lumen of both the small and large intestines was flushed with 5-8 ml of PBS and the contents were gently squeezed out. They were homogenized and were finally adjusted to 10 ml with PBS. All the Evan's blue from both the gut contents and from the serum was precipitable with 10% trichloracetic acid which indicated that the dye was bound to protein and was not transuding into the gut lumen as free dye. That the dye was bound to protein was further established by immunoelectrophoresis where it was found that the dye both from serum and gut contents migrated with the albumin peak and not as free dye. Moreover, it was found that the dye in the gut contents was not dialysable. In order to extract Evan's blue and to remove turbidity, 2 ml of acetone was added to an equal volume of homogenate, (Allen, 1951) was mixed well, and was centrifuged at 4000 rpm for 8 min. The optical density (O.D.) of the clear supernatant was immediately measured

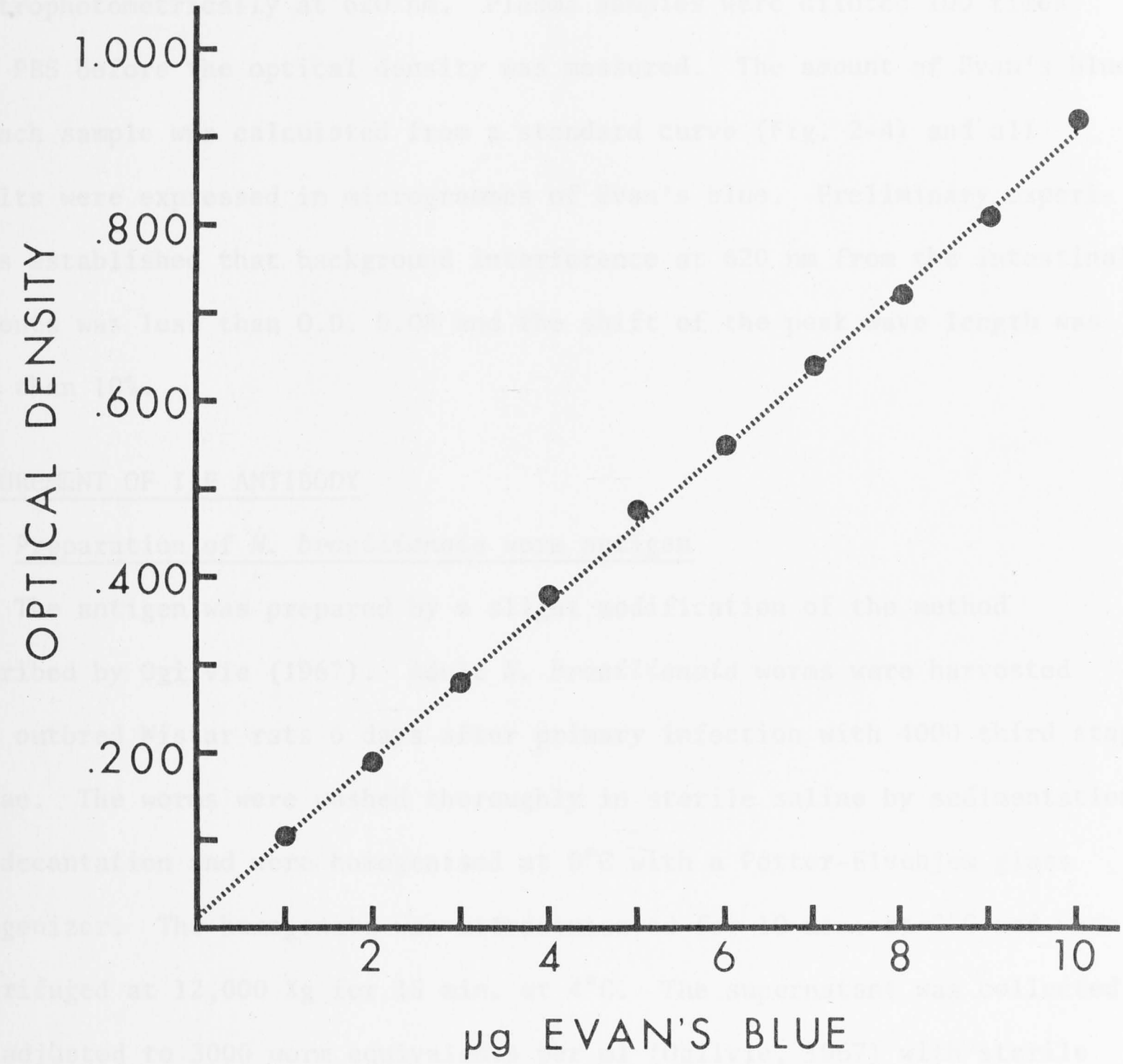


Fig. 2-4: Standard curve for spectrophotometrical measurement of Evan's blue. Optical densities were measured in a Hitachi Spectrophotometer Model 101 at 620 nm in cuvettes with a 1 cm path length. Linearity was obtained at concentrations up to 10 µg Evan's blue per millilitre of PBS.
Slope: 0.0893. Intercept: 0.016.

spectrophotometrically at 620 nm. Plasma samples were diluted 100 times with PBS before the optical density was measured. The amount of Evan's blue in each sample was calculated from a standard curve (Fig. 2-4) and all results were expressed in microgrammes of Evan's blue. Preliminary experiments established that background interference at 620 nm from the intestinal contents was less than O.D. 0.08 and the shift of the peak wave length was less than 10%.

MEASUREMENT OF IgE ANTIBODY

Preparation of *N. brasiliensis* worm antigen

The antigen was prepared by a slight modification of the method described by Ogilvie (1967). Adult *N. brasiliensis* worms were harvested from outbred Wistar rats 6 days after primary infection with 4000 third stage larvae. The worms were washed thoroughly in sterile saline by sedimentation and decantation and were homogenised at 0°C with a Potter-Elvehjem glass homogenizer. The homogenate was ultrasonicated for 10 min. at 0°C and was centrifuged at 12,000 Xg for 15 min. at 4°C. The supernatant was collected and adjusted to 3000 worm equivalents per ml (Ogilvie, 1967) with sterile saline and kept at -70°C before use.

Homologous passive cutaneous anaphylaxis (PCA)

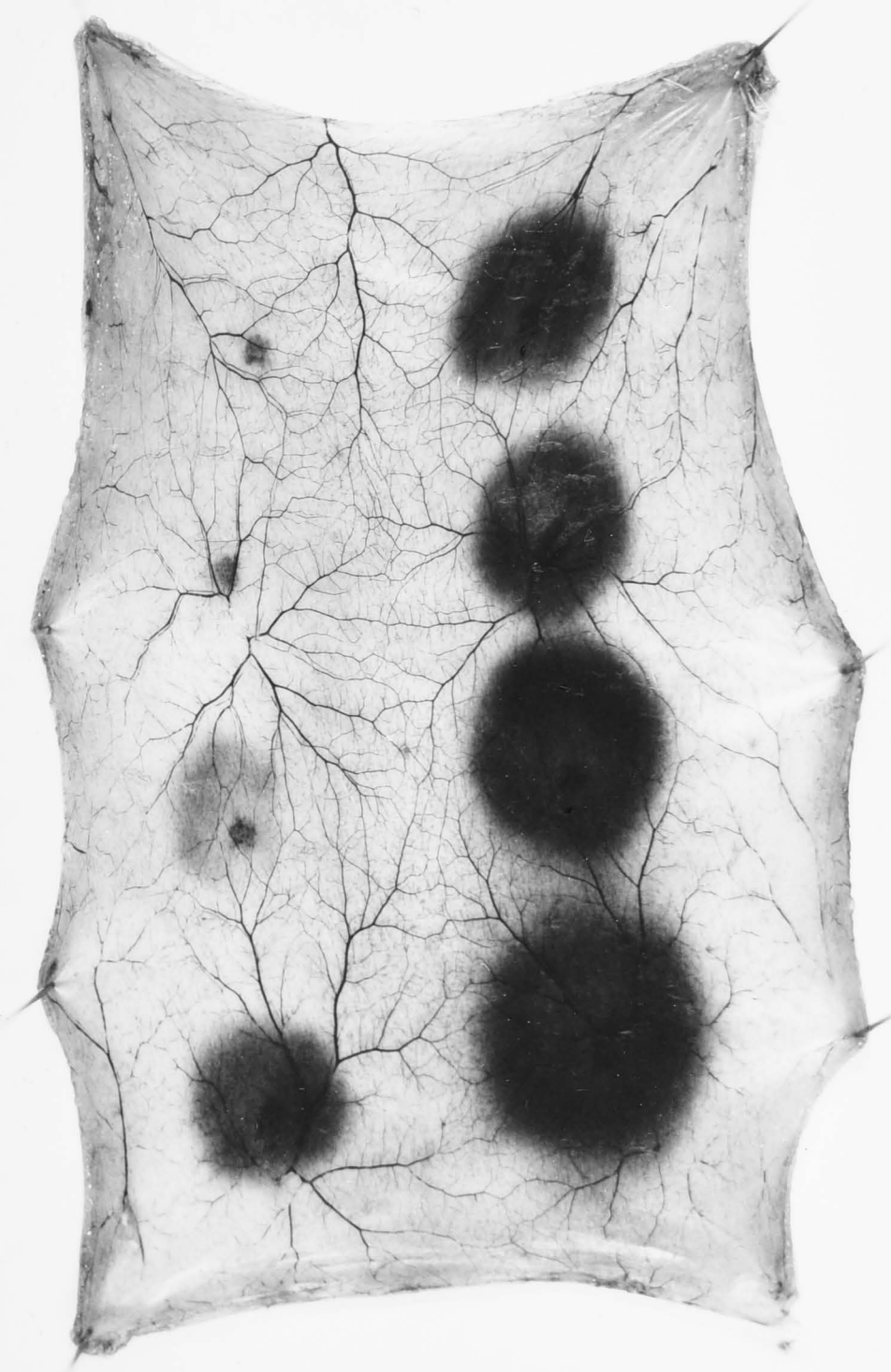
The level of circulating IgE antibody against *N. brasiliensis* was assayed by PCA as described by Ovary (1964).

One-tenth of a millilitre from serial two-fold dilutions of serum were intradermally injected into 11-13 week old female outbred Wistar rats. Seventy-two hours later the rats were injected intravenously with 0.5 ml of worm antigen (adjusted to 2000 worm equivalent per ml just before use) and 0.5 ml of 1% Evan's blue in saline. The minimal dose of antigen which caused a maximal skin reaction was determined by a preliminary titration

Fig. 2-5: The result of a typical homologous passive cutaneous anaphylaxis (PCA) assay in a rat. A normal Wistar rat was injected intradermally with 0.1 ml samples of two-fold dilutions of 10 times diluted rat serum obtained 1 week after tertiary infection with *N. brasiliensis*. Seventy-two hours later, 0.5 ml of worm extract (2000 worm equivalent/ml; see this Chapter) and 0.5 ml of 1% Evan's blue were injected intravenously. Extravasation of Evan's blue on the under-surface of the skin was examined 30 min. after challenge.

Rat homologous PCA

Ag; N. brasiliensis



experiment. The highest dilutionsof serum giving a blue spot greater than 5mm in diameter were determined by killing the rats and examining the under-surface of the skin 30 min after antigen challenge. All the PCA titres are expressed as reciprocals.

A typical PCA titration is shown in Fig. 2-5.

STATISTICAL CALCULATIONS

Standard error of a mean (SE)

This was calculated by following formula:

$$SE = \frac{\sigma}{\sqrt{n}}$$

where σ is the standard deviation:

$$\sigma = \sqrt{\frac{\Sigma(\overline{X} - X)^2}{n-1}}$$

n: number of observations

X: the individual values of the observations

$\overline{X}$: arithmetic mean of the series of observations

Student's t-test

The probability that the difference represents chance variation was estimated by using Student's t-test. The formula used to calculate t is:

$$t = \sqrt{\frac{|\overline{X} - \overline{Y}|}{\frac{(n_x + n_y)\ [(n_x-1)\sigma_x^2 + (n_y-1)\sigma_y^2]}{n_x n_y\ (n_x + n_y - 2)}}}$$

n_x, n_y: number of observations in each group

$\overline{X}$, $\overline{Y}$: arithmetic mean of the series of values in each group

σ_x σ_y: the standard deviation for each group.

The probability for the given t value was found in a table (Steel and Torrie, 1960) where the degree of freedom was $(n_x + n_y - 2)$. When the P value is < 0.05, the difference is considered to be 'statistically significant' and when the P value is < 0.01, the difference is 'highly significant'.

Linear regression

When a straight line is to be fitted to data consisting of more than two pairs of values, the line which best fits the data must be calculated. The regression equation $Y = a + bX$ was determined by using the following formula:

Regression coefficient $\quad b = \frac{\Sigma xy}{\Sigma x^2}$

where $x = X-\overline{X}, \quad y = Y-\overline{Y}$

X,Y: individual values of observation

$\overline{X},\overline{Y}$: mean of Xs of Ys

Y intercept $\quad a = \overline{Y} - b\overline{X}$

All calculations were done using the computer terminal in the Department of Immunology, JCSMR and the programmes were prepared by the Computer Centre.

CHAPTER 3

PROTECTION AGAINST *N. BRASILIENSIS* BY ADOPTIVE IMMUNIZATION WITH IMMUNE THORACIC DUCT LYMPHOCYTES (TDL)

CHAPTER 3

PROTECTION AGAINST *N. BRASILIENSIS* BY ADOPTIVE IMMUNIZATION WITH IMMUNE THORACIC DUCT LYMPHOCYTES (TDL)

The immune expulsion of *N. brasiliensis* is thought to be a multiphasic (Kelly, 1973) or two-step (Ogilvie and Love, 1974) process in which the adult parasites must first be damaged by humoral antibodies before they can be expelled non-specifically by a lymphocyte-mediated step (see Chapter 1). Although there is much circumstantial evidence that antibodies can damage adult parasites in the gut lumen (Ogilvie and Hockley, 1968) the evidence that the worms are expelled non-specifically by the action of lymphocytes is less clear-cut. Keller and Keist (1972) demonstrated that the adoptive transfer of MLNC obtained from normal rats into lightly irradiated (400 r) rats caused the accelerated expulsion of implanted 'damaged' worms but Dineen, Kelly and Love (1973) were unable to confirm this observation. The latter group showed that adoptive immunization with immune MLNC caused the expulsion of implanted 'damaged' worms but had little effect on 'normal' adult worms. They also emphasized the importance of a bone marrow-derived component in the non-specific expulsive phase (Dineen and Kelly, 1973; Kelly *et al.* 1973).

Recent experiments by Ogilvie *et al.* (1977) suggest that MLNC may be a poor source of protection when compared with TDL. Moreover, they also found that cells separated from immune TDL and which lacked sIg were able to expel implanted 'damaged' worms from heavily (750 rad) irradiated rats. These observations refute the proposal that a myeloid component is required for worm expulsion.

In view of these conflicting results, there is some doubt as to the validity of the multiphasic or two-step hypothesis. It could be argued, for example, that the protective capacity of different lymphocyte populations is related to the relative content of effector cells and that the different

susceptibilities of 'damaged' and 'normal' worms to adoptive protection is a quantitative rather than a qualitative phenomenon. The experiments in this chapter were, therefore, designed to examine several of the parameters which govern the ability of immune lymphocytes to protect against *N. brasiliensis*. The protective capacity of different sources of immune cells were tested against larval infections and against implanted 'normal' and 'damaged' worms. The results show that TDL drained from donors on the tenth day of infection are highly effective not only against larval infections and 'damaged' worms, but also against 'normal' worms.

RESULTS

Worm burden kinetics after infection with 4000 L_3 or 1000 L_3

$(PvG/c \times DA)F_1$ cell donors and recipients were infected with 4000 and 1000 L_3 respectively. 'Normal' and 'damaged' adult worms were collected 6 and 11 days respectively after primary infection of $(PvG/c \times DA)F_1$ rats with 4000 L_3. These parameters were chosen after examining the worm burden kinetics during infection with 4000 and 1000 L_3 (Fig. 3-1). Approximately 70% of the injected larvae established as adults in the intestine with both doses. The worm burden was stable 8 days after infection with 1000 L_3 and was slightly decreased by day 10,with a rapid phase of expulsion occurring between days 10 and 12. On the other hand, up to 90% of the established parasites were gradually expelled between days 6 and 12 after infection with 4000 L_3, and the remaining worms were rapidly expelled between days 12 and 14. With both doses of infective larvae, the final expulsion of adult worms was rapid (within 48h) and complete so that no residual worms were found after day 14.

Immunological status of the cell donor

To investigate at what stage of infection the effector cells appear in the donor's circulating pool of lymphocytes, TDL were drained from donor

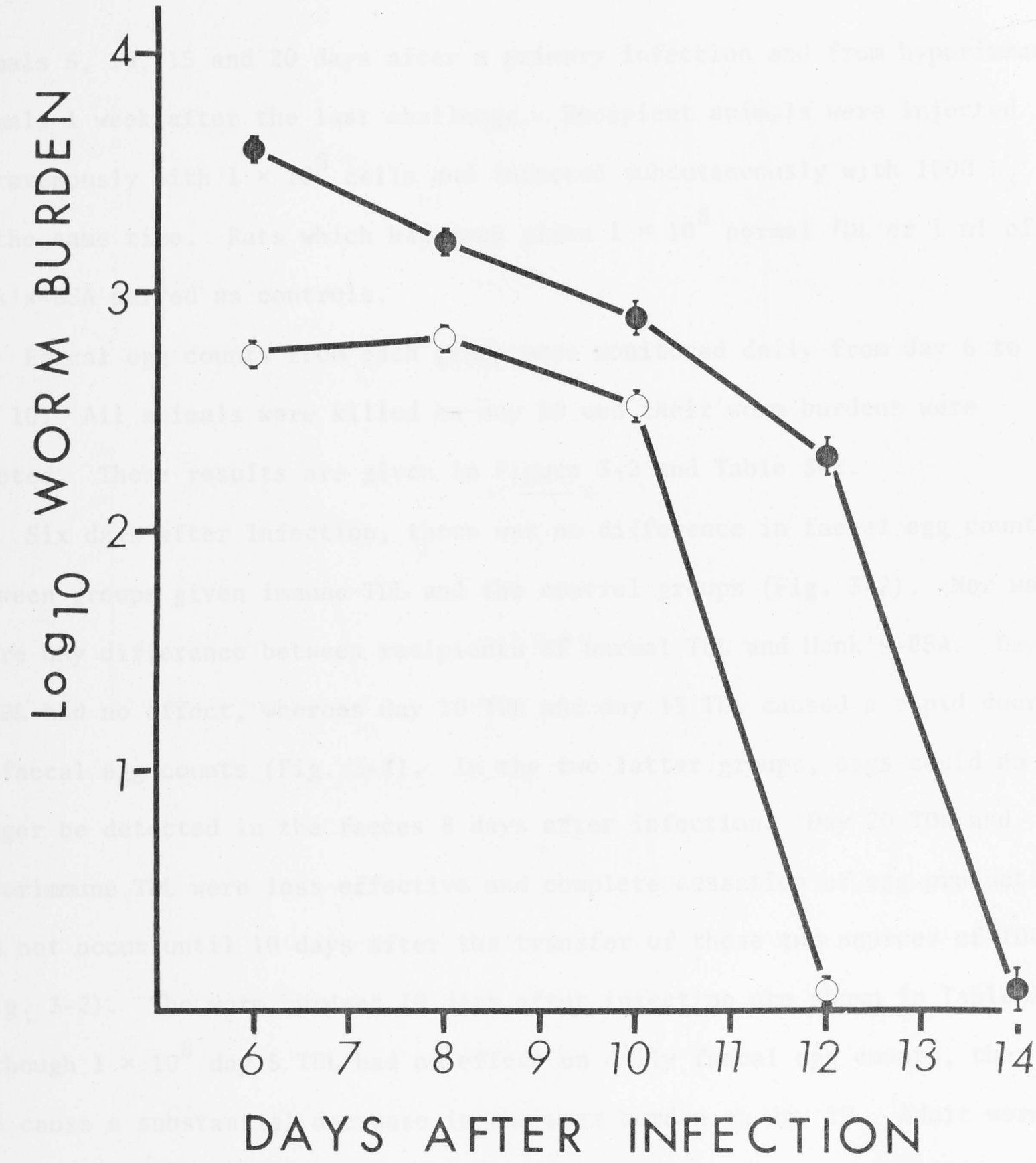


Fig. 3-1: Worm burden kinetics in (PvG/c × DA)F_1 rats infected with 1000 L_3 (o —— o) or with 4000 L_3 (• —— •). Each point represents $\log_{10}$ geometric mean ± standard error for a group of 5 rats.

animals 5, 10, 15 and 20 days after a primary infection and from hyperimmune animals 1 week after the last challenge. Recipient animals were injected intravenously with 1×10^8 cells and infected subcutaneously with 1000 L_3 at the same time. Rats which had been given 1×10^8 normal TDL or 1 ml of Hank's-BSA served as controls.

Faecal egg counts from each group were monitored daily from day 6 to day 10. All animals were killed on day 10 and their worm burdens were counted. These results are given in Figure 3-2 and Table 3-1.

Six days after infection, there was no difference in faecal egg counts between groups given immune TDL and the control groups (Fig. 3-2). Nor was there any difference between recipients of normal TDL and Hank's-BSA. Day 5 TDL had no effect, whereas day 10 TDL and day 15 TDL caused a rapid decrease in faecal egg counts (Fig. 3-2). In the two latter groups, eggs could no longer be detected in the faeces 8 days after infection. Day 20 TDL and hyperimmune TDL were less effective and complete cessation of egg production did not occur until 10 days after the transfer of these two sources of TDL (Fig. 3-2). The worm burdens 10 days after infection are shown in Table 3-1. Although 1×10^8 day 5 TDL had no effect on daily faecal egg counts, they did cause a substantial decrease in the worm burden on day 10. Adult worms were completely expelled in recipients given day 10 or day 15 TDL and more than 90% of the parasites were expelled by day 10 in recipients given day 20 TDL or hyperimmune TDL (Table 3-1).

Worm burden kinetics after adoptive immunization with day 10 and hyperimmune TDL

To further compare the efficacy of day 10 and hyperimmune TDL, 1×10^8 cells were injected into rats which were infected with 1000 L_3 at the same time. Infected rats given Hank's-BSA served as controls. Groups of 5 rats were killed at 48 h intervals and their worm burdens are shown in Figure 3-3.

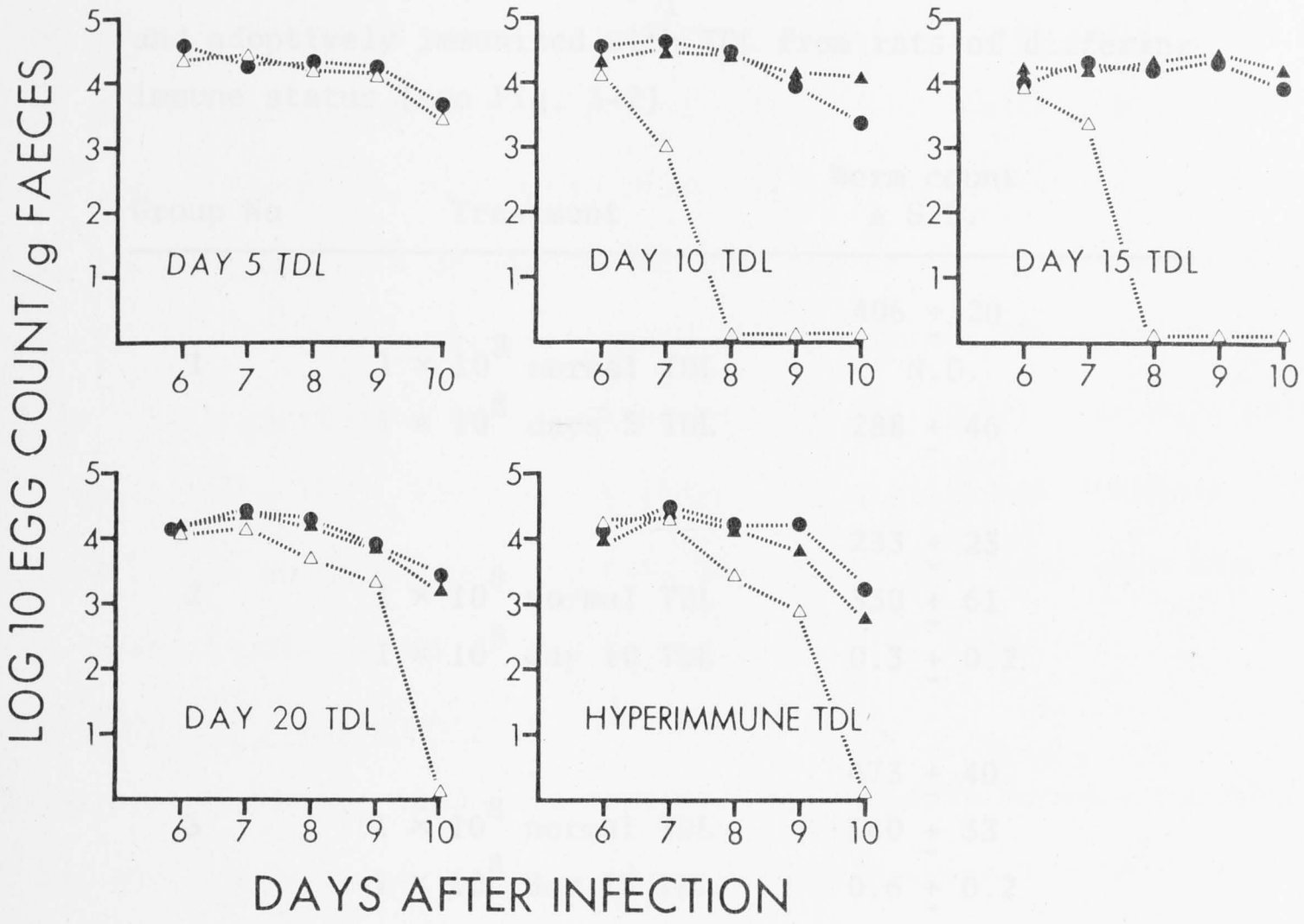


Fig. 3-2: Daily faecal egg count from (PvG/c × DA)F_1 rats infected with 1000 L_3 and given 1×10^8 normal (▲---▲) or immune (Δ---Δ) TDL obtained from donors on different days (5, 10, 15 and 20) after primary infection or 1 week after a tertiary infection (hyperimmune). Control animals were not given cells (● --- ●). Data points represent $\log_{10}$ daily faecal egg counts for each group.

Table 3-1: Worm burdens in (PvG × DA)F_1 rats infected with 1000 L_3 and adoptively immunized with TDL from rats of differing immune status (see Fig. 3-2)

Group No	Treatment		Worm count ± S.E.
1	-		406 ± 20
	1×10^8	normal TDL	N.D.
	1×10^8	days 5 TDL	288 ± 46
2	-		233 ± 23
	1×10^8	normal TDL	330 ± 61
	1×10^8	day 10 TDL	0.3 ± 0.2
3	-		473 ± 40
	1×10^8	normal TDL	540 ± 33
	1×10^8	day 15 TDL	0.6 ± 0.2
4	-		274 ± 41
	1×10^8	normal TDL	332 ± 20
	1×10^8	day 20 TDL	19 ± 12
5	-		413 ± 32
	1×10^8	normal TDL	244 ± 58
	1×10^8	hyperimmune TDL	11 ± 4

Worm burdens were counted 10 days after infection. Each value represents the mean ± standard error for a group of 5 rats. N.D. : not done.

In the controls the worm burden remain unchanged between days 6 and 8, but about 30% of the worms were expelled by day 10 with the final rapid phase of expulsion occurring between days 10 and 12 (Fig. 3-3). In the recipients of 1×10^8 hyperimmune TDL, the worm burden was one third that of the controls by day 8, and more than 95% of worms were expelled by day 10. There was no difference on day 6 between the recipients of hyperimmune TDL and the controls (Fig. 3-3). As suggested by the previous results, day 10 TDL were more effective than hyperimmune TDL. There was a slight but significant decrease (30%) in the worm burden on day 6 and more than 95% of the parasites were expelled by day 8. Worm expulsion was complete by day 10 (Fig. 3-3).

Dose-response relationship

To determine whether there was a direct relationship between the transfer of TDL and the expulsion of the parasites, a range between 1×10^7 and 4×10^8 hyperimmune and day 10 TDL were injected into recipients which were infected with 1000 L_3 at the same time. Control animals were injected with Hank's-BSA. All animals were killed on day 8 and the worm burdens were counted (Fig. 3-4).

The efficacy of the transferred cells was dose dependent, day 10 TDL being more effective than hyperimmune TDL (Fig. 3-4). Thus, 1×10^7 day 10 TDL caused a significant decrease (27.7%) in the worm burden whereas 1×10^7 hyperimmune TDL had no effect. Inverse and linear relationships between the number of cells transferred and the worm burdens occurred at doses between 5×10^7 and 4×10^8 cells with both sources of TDL. Moreover, it is clear that day 10 TDL are more effective than hyperimmune TDL in this dose range (Fig. 3-4).

Comparison between MLNC and TDL

Since previous data describing the adoptive response against *N. brasiliensis* have been obtained by the transfer of MLNC (Rev. by Ogilvie and Love, 1974) the effects of adoptive immunization with day 10 TDL and

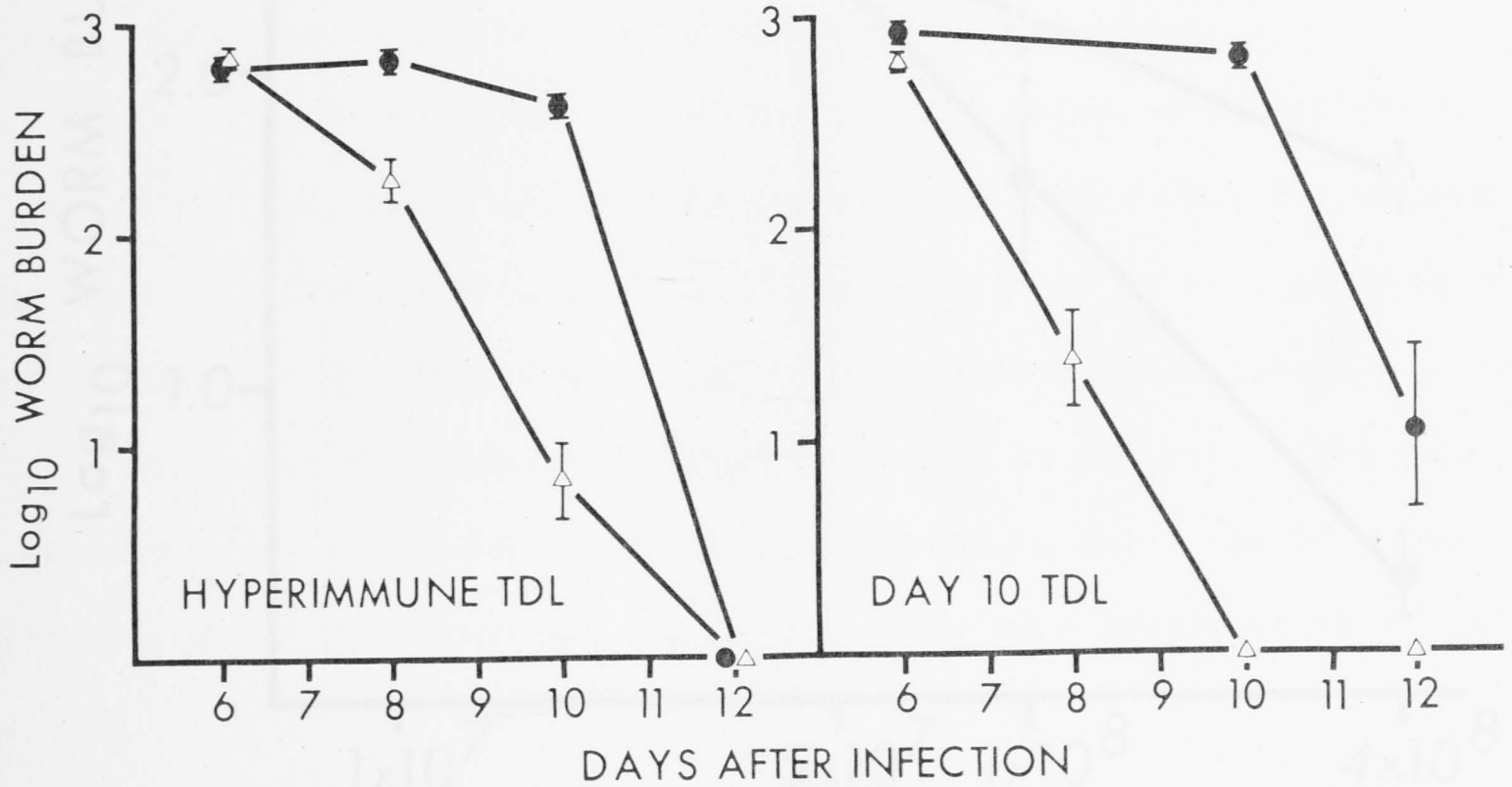


Fig. 3-3: Worm burden kinetics in (PvG/c × DA)F_1 rats infected with 1000 L_3 and adoptively immunised with hyperimmune TDL or with day 10 TDL at the time of infection. left: 1×10^8 hyperimmune TDL (Δ —— Δ); right: 1×10^8 day 10 TDL (Δ —— Δ). Infected controls were not given cells (● —— ●). Data points show the $\log_{10}$ geometric mean ± standard error for groups of 5 rats.

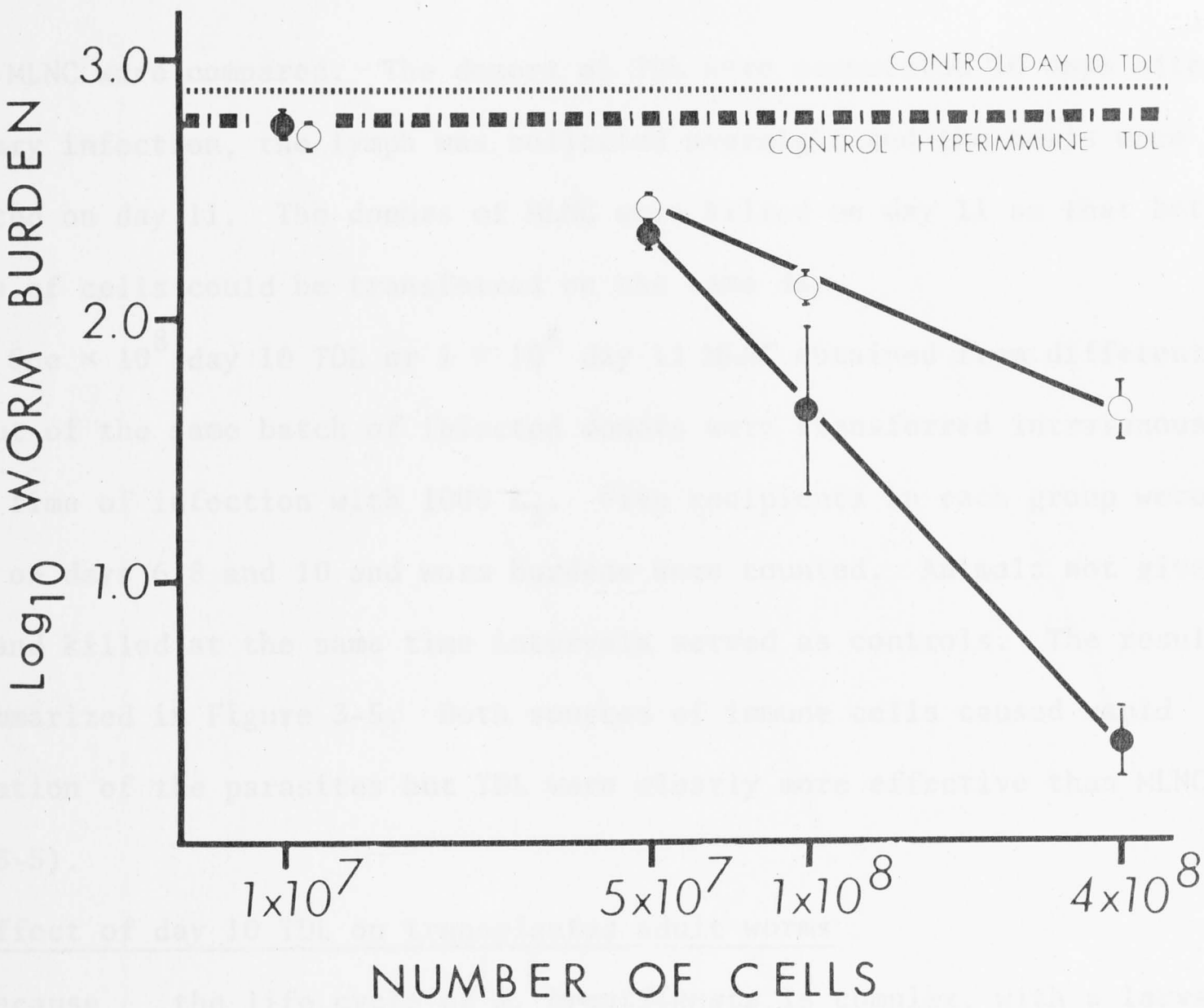


Fig. 3-4: A logarithmic plot of the dose-response relationship between the worm burden and the number of immune TDL transferred.

(PvG/c × DA)F_1 rats were infected with 1000 L_3 and, at the same time, were given different doses of hyperimmune (o —— o) or day 10 (• —— •) TDL. Worm burdens were counted 8 days after infection and cell transfer. Each point represents $\log_{10}$ geometric mean ± standard error of the worm burden for a group of 5 rats. Log_{10} geometric mean ± standard error of the worm burdens in rats which were infected but which were not given cells is represented by (.....) for the control for day 10 TDL and by (— · — · —) for the control for hyperimmune TDL.

day 11 MLNC were compared. The donors of TDL were cannulated 10 days after a primary infection, the lymph was collected overnight and the cells were harvested on day 11. The donors of MLNC were killed on day 11 so that both sources of cells could be transferred on the same day.

One $\times 10^8$ day 10 TDL or 1×10^8 day 11 MLNC obtained from different rats out of the same batch of infected donors were transferred intravenously at the time of infection with 1000 L_3. Five recipients in each group were killed on days 6, 8 and 10 and worm burdens were counted. Animals not given cells and killed at the same time intervals served as controls. The results are summarized in Figure 3-5. Both sources of immune cells caused rapid elimination of the parasites but TDL were clearly more effective than MLNC (Fig. 3-5).

Effect of day 10 TDL on transplanted adult worms

Because the life cycle of *N. brasiliensis* is complex, with a larval migratory phase, it was important to establish the role of transferred lymphocytes against adult worms more directly. Six hundred adult worms obtained from (PvG/c × DA)F_1 rats on day 6 ('normal' worms) or on day 11 ('damaged' worms) of a 4000 L_3 primary infection, were implanted intraduodenally into normal recipients which were intravenously injected with different doses of day 10 TDL.

The worm burden kinetics were examined and it was found that when 600 'normal' worms were transplanted intraduodenally into normal rats about 80% (437 ± 76) of the original burden was present on day 7 (Table 3-2). On the other hand, in those rats given 1×10^8 day 10 TDL at the time of adult worm implantation, 85% of the worms were expelled by day 5 and there was complete expulsion by day 7 (Table 3-2).

The dose-response relationship was examined on day 5 of this experiment and the results are summarized in Table 3-3. One, 2 and 4×10^8 day 10 TDL all caused worm expulsion and 4×10^8 cells were the most effective,

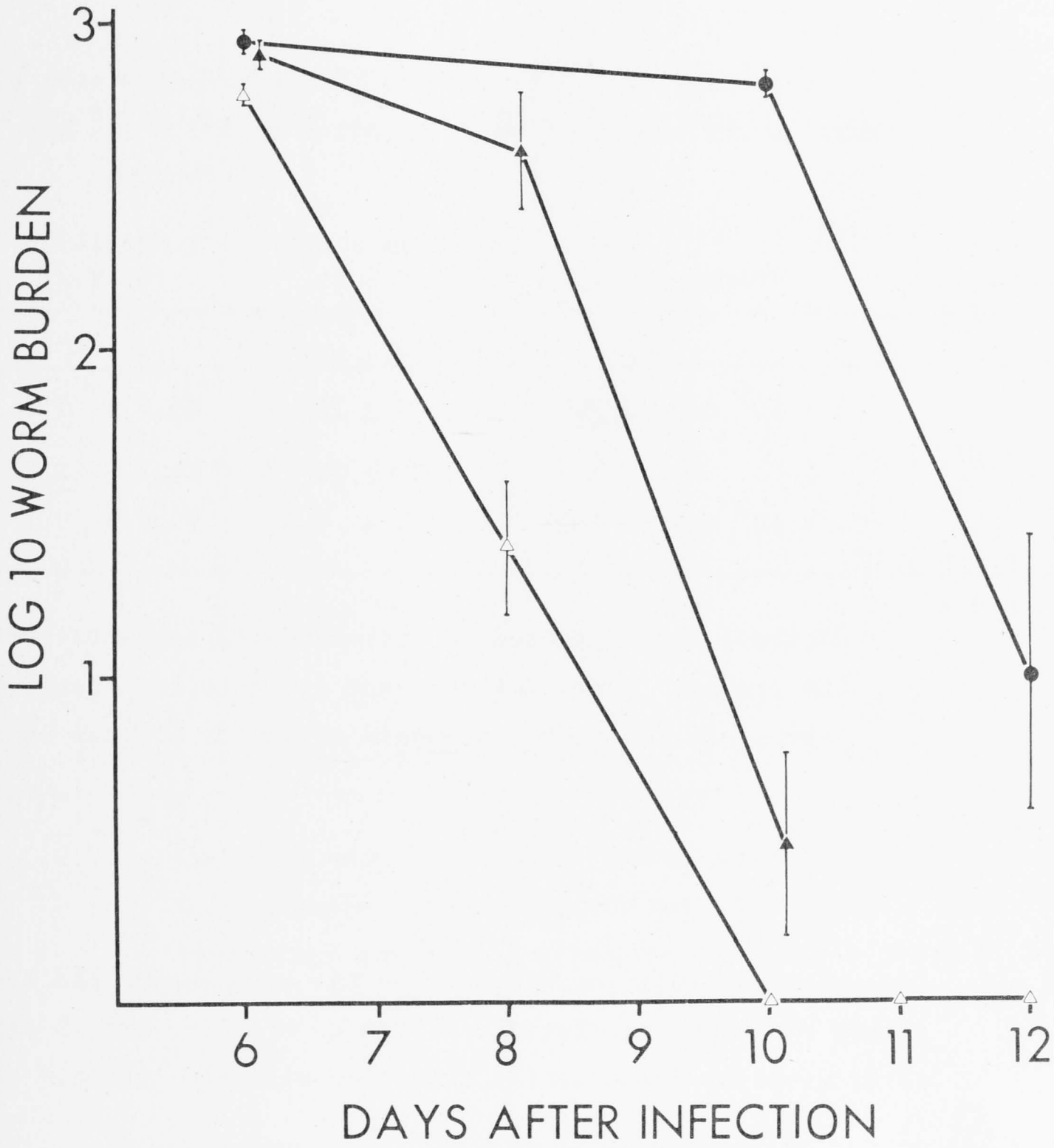


Fig. 3-5: Worm burden kinetics in (PvG/c × DA)F_1 rats infected with 1000 L_3 and, at the same time, adoptively immunised with 1×10^8 day 10 TDL (△ —— △) or with 1×10^8 day 11 immune mesenteric lymph node cells (▲ —— ▲). Infected controls were not given cells (● —— ●). Each point represents $\log_{10}$ geometric mean ± standard error of the worm counts for a group of 5 rats.

Table 3-2: Worm burden kinetics in (PvG × DA)F_1 rats infected with 600 'normal' worms and adoptively immunized with 1×10^8 day 10 TDL.

Days after transfer	Treatment	Worm count ± S.E.	% Expulsion ± S.E.
day 5	-	558 ± 30	
	1×10^8 day 10 TDL	82 ± 29	85.3 ± 5.2
day 7	-	437 ± 76	21.8 ± 13.7
	1×10^8 day 10 TDL	0.5 ± 0.5	99.9 ± 0.1

Controls were infected by intraduodenal implantation of 600 'normal' worms but were not given cells. Each value represents the mean ± standard error for a group of 5 rats.

Table 3-3: Dose-response relationship between the worm burden and the number of cells transferred in (PvG/c × DA)F_1 rats 5 days after adoptive immunization infection with 600 'normal' worms.

No. of cells	Worm count ± S.E.	% Expulsion ± S.E.
-	558 ± 30	
1×10^8 day 10 TDL	82 ± 29	85.3 ± 5.2
2×10^8 day 10 TDL	132 ± 62	76.4 ± 11.1
4×10^8 day 10 TDL	23 ± 14	96.0 ± 2.4

Each value represents the mean ± standard error for a group of 5 rats.

Table 3-4: Worm burden kinetics in (PvG/c × DA)F_1 rats infected with 600 'damaged' worms and adoptively immunized with 1×10^8 day 10 TDL

Days after transfer	Treatment	Worm count ± S.E.	% Expulsion ± S.E.
day 3	-	480 ± 63	
	1×10^8 day 10 TDL	261 ± 22	45.7 ± 4.6
day 5	-	31 ± 11	93.6 ± 2.4
	1×10^8 day 10 TDL	2 ± 1	99.5 ± 0.3
day 7	-	4 ± 4	99.3 ± 0.7
	1×10^8 day 10 TDL	0 ± 0	100.0 ± 0

Controls were infected by intraduodenal implantation of 600 'damaged' worms but were not given cells. Each value represents the mean ± standard error for a group of 5 rats.

Table 3-5: Dose response relationship between the worm burden and the number of cells transferred in (PvG/c × DA)F_1 rats 3 days after adoptive immunization and infection with 600 'damaged' worms.

No. of cells	Worm count ± S.E.	% Expulsion ± S.E.
-	480 ± 63	
1×10^7 day 10 TDL	255 ± 14	46.9 ± 3.0
1×10^8 day 10 TDL	261 ± 22	45.7 ± 4.6
4×10^8 day 10 TDL	71 ± 9	85.3 ± 1.9

Each value represents the mean ± standard error for a group of 5 rats.

resulting in 96% expulsion (Table 3-3). The worm burdens found in the recipients of 1×10^8 and 2×10^8 cells were not significantly different.

Table 3-4 shows the worm burden kinetics of recipients given 600 'damaged' worms intraduodenally with and without the intravenous transfer of 1×10^8 day 10 TDL. More than 90% of the transplanted 'damaged' worms were expelled by day 5 in recipients given no cells. In this experimental system, transfer of 1×10^8 day 10 TDL caused a significant decrease in the worm burden by day 3 and almost complete expulsion of parasites occurred by day 5. The dose-response relationship was examined on day 3. Results in Table 3-5 shows that even with 1×10^7 day 10 TDL there was partial expulsion in the 'damaged' worms and that 4×10^8 cells caused expulsion of 85% of the worm burden. There was, however, no difference in the worm burdens between recipients of 1×10^7 and 1×10^8 TDL on day 3 (Table 3-5).

DISCUSSION

These experiments establish that circulating lymphocytes obtained from donors on the tenth day of a primary infection provide a high degree of protection against the adult intestinal stage of *N. brasiliensis*. They have, in addition, shown that there is a direct and inverse relationship between the number of cells transferred and the number of parasites remaining in the gut lumen after a larval infection. It is also clear that TDL obtained from rats just prior to, and during, the rapid phase of worm expulsion in a primary infection are more protective than MLNC obtained at this time and than TDL drained from hyperimmune donors. These results emphasise the importance of kinetic studies and of dose-response experiments in evaluating the efficacy of transferred cells.

Although others (see Introduction and also Chapter 1) have argued that the worms must first be damaged by antibody before they can be expelled non-specifically by the action of lymphocytes, the present results show that

day 10 TDL conferred a high degree of protection not only against adult worms established by larval infection, but also against intra-duodenally implanted 'normal' and 'damaged' worms. In addition, it is clear from the kinetic studies of the 'damaged' worms that even in normal rats such worms are rapidly expelled. These results suggest that the different susceptibilities of 'normal' and 'damaged' worms to the transfer of immune lymphoctyes is a quantitative rather than qualitative difference and that the multiphasic or two-step theory of worm expulsion (Kelly, 1973; Ogilvie and Love, 1974) may need to be re-evaluated using TDL rather than MLNC as a source of immune lymphocytes.

There may be several reasons why different cell sources should vary in their protective capacities. For example, the proportion of effector cells, or their immediate precursors, is probably higher in day 10 TDL than in hyperimmune TDL. In support of this, day 10 TDL are able to confer the intestinal mast cell response more rapidly than hyperimmune TDL (Chapter 5). It is also possible that the predominant effector cells in hyperimmune TDL are memory cells which would require a lag phase during which they divide and differentiate before they can cause worm expulsion. The relatively poor protective capacity of day 11 MLNC when compared with day 10 TDL may be because mesenteric nodes lack effector cells and/or contain a high proportion of non-recirculating cells which probably do not re-localize in the small intestine.

TDL have the capacity to adoptively transfer both humoral antibody and cell-mediated responses and, additionally, they carry immunological memory (Gowans and McGregor, 1965). It is possible, therefore, that one or several of these capacities enable immune TDL to confer protection against *N. brasiliensis*. For example, the major source of specific IgA antibody-forming cells in the gut mucosa of primed rats after oral boosting was shown to be TDL (Pierce and Gowans, 1975). This capacity of TDL to furnish

antibody-forming cell precursors to the gut mucosa together with the reported occurrence of specific IgA antibody in the gut lumen of rats infected with *N. brasiliensis* (Poulain *et al.* 1976b) raises the possibility that one effect of adoptive immunization with TDL is to transfer a specific IgA antibody response. However, this possibility would need to be reconciled with the results of Ogilvie *et al.* (1977) who reported that implanted 'damaged' worms can be expelled from heavily irradiated (750 rad) rats by a subpopulation of TDL which lack sIg. These conflicting possibilities will be examined in the chapters that follow.

CHAPTER 4

THE PROTECTIVE CAPACITIES OF FRACTIONATED IMMUNE TDL.

CHAPTER 4

THE PROTECTIVE CAPACITIES OF FRACTIONATED IMMUNE TDL

The immune expulsion of the nematode *Nippostrongylus brasiliensis* from rat intestine can be accelerated by the adoptive transfer of immune MLNC (Kelly and Dineen, 1972; Dineen, *et al.*, 1973b). In Chapter 3 it was shown that TDL are also highly protective against the intestinal phase of this parasite and that (a) the immune status of the cell donor is important, the most effective cell populations being obtained 10 days after primary infection; (b) these cells confer a high degree of protection not only against adult worms established by larval infection but also against intraduodenally transplanted 'normal' and 'damaged' adult worms; (c) there is a direct and inverse relationship between the number of cells transferred and the number of parasites remaining in the gut lumen.

Even though *N. brasiliensis* infection causes severe pathological changes in the intestine (Symons, 1965), the parasites remain on the surface of the mucosa and do not penetrate the epithelium (Symons, 1976). This observation raises important questions as to how lymphoid cells can exert a direct effect on the parasites. Most workers agree that specific antibody has an important role (Rev. by Ogilvie and Love, 1974; Rev. by Jarrett and Urquhart, 1971; Wilson and Bloch, 1968) although it is generally thought that the final phase of expulsion is brought about by non-specific mediators (Rev. by Ogilvie and Love, 1974; Rev. by Kelly, 1973).

Lymphocytes drained from the rat thoracic duct can transfer a variety of immune responses into irradiated recipients (Gowans and McGregor, 1965) and the plasma cells in the lamina propria of the rat intestine are derived, to some extent, from large lymphocytes found in TDL (Gowans and Knight, 1964; Griscelli *et al.*, 1969; Hall *et al.*, 1972). Recently Pierce and Gowans

(1975) found that after intra-intestinal challenge with cholera toxin, the major source of specific antibody forming cells in the gut mucosa was thoracic duct lymphocytes. Most of these antibody-forming cells, both in lymph and in the lamina propria, contained IgA. These findings, together with the observation in Chapter 3 on the abilities of immune TDL to protect against *N. brasiliensis* suggest that cell fractionation techniques may shed further light on the cellular mechanisms involved in worm expulsion.

Lymphocytes bearing surface Ig(sIg^+ cells) can be separated from cells lacking sIg(sIg^- cells) using a rosetting technique developed by Parish and Hayward (1974a). Moreover, the distribution and functions of different lymphocyte subpopulations in rat thoracic duct lymph have been well characterized by this technique (Parish and Hayward, 1974b, c; Mason, 1976).

In this chapter, immune TDL obtained from donors infected with *N. brasiliensis* were fractionated into sIg^+ and sIg^- cells and the abilities of each subpopulation in the adoptive response against *N. brasiliensis* were examined. It was found, in agreement with Ogilvie *et al.* (1977), that the protective capacity of day 10 TDL resided exclusively in the sIg^- population. Similarly, the major protective capacity of hyperimmune TDL was associated with sIg^- cells although slight, but significant, protection was also conferred by sIg^+ hyperimmune TDL. These results are discussed with regard to the mucosal changes associated with worm expulsion.

RESULTS

Efficacy of separation of sIg^+ and sIg^- cells

Surface and intracellular immunoglobulins of normal and immune TDL were examined by the direct and indirect immuno-peroxidase techniques both prior to, and after separation in order to determine the efficacy of the fractionation procedure. The results are summarized in Table 4-1. Similar proportions of both normal and immune TDL rosetted for sIg and these values

Table 4-1: Efficacy of the fractionation of TDL into sIg^- and sIg^+ subpopulations by the rosetting technique

		No. of cells applied	No. of cells recovered
Normal TDL	Original†	24×10^7	
	Upper layer*		8.9×10^7
	Lower layer**		11.4×10^7
Day 10 TDL (Exp. 1)	Original	16×10^8	
	Upper layer		5.0×10^8
	Lower layer		4.5×10^8
Day 10 TDL (Exp. 2)	Original	20×10^8	
	Upper layer		7.9×10^8
	Lower layer		5.3×10^8
HITDL (1w)	Original	20×10^8	
	Upper layer		7.1×10^8
	Lower layer		8.8×10^8
HITDL (5w)	Original	20×10^8	
	Upper layer		8.6×10^8
	Lower layer		6.9×10^8

†: original unseparated population of TDL

*, **: Cells recovered from upper layer and lower layer respectively after separation on Isopaque/Ficoll. Upper layer: non-rosetting lymphocytes = sIg^-; lower layer: rosetted lymphocytes = sIg^+

ND: Not done.

a total recovery is expressed as the percentage of the total original cell population before it was applied to Isopaque/Ficoll.

b the percentage of sIg^+ cells in each population was counted after labelling the cells with the immunoperoxidase technique.

c intracellular immunoglobulins were detected by the immunoperoxidase technique and the percentage of Ig-synthesising cells in each population is recorded.

Relative ratio (%)	Total recovery[a] (%)	sIg$^+$ by immuno-peroxidase[b] (%)	Intracellular Ig by immuno-peroxidase[c] (%)
		45.5	1.2
43.8	84.6	3.3	4.1
56.2		90.7	2.0
		41.6	2.2
52.6	59.4	0.1	1.7
47.4		N.D.	1.8
		39.2	1.9
60.0	66.0	0.1	2.3
40.0		N.D.	1.8
		46.7	6.1
45.0	79.5	0.3	2.1
55.0		N.D.	4.7
		43.5	1.5
55.7	77.5	1.6	1.5
44.3		96.7	1.2

were in good agreement with the proportions of sIg^+ cells in the original cell populations detected by the immunoperoxidase technique (Fig. 4-1a). Although there was some loss of cells during the separation procedure, there was reasonably good agreement between the proportion of sIg^+ cells recovered after rosetting and the proportion of sIg^+ cells in the original population as detected by the immunoperoxidase technique. This would suggest that the loss of cells was not selective and that it was occurring in both sub-populations.

The efficacy of the separation technique is shown by the very low proportions of sIg^+ cells observed (Fig. 4-1b) in the upper layers after each separation (Table 4-1). However, the separation of Ig-synthesising cells was not complete, and a significant proportion of such cells was invariably demonstrated amongst the sIg^- cells (Table 4-1, Fig. 4-1c) as well as amongst the sIg^+ cells (Fig. 4-1d).

The effect of cell separation on the adoptive response

Thoracic duct lymphocytes drained from donor rats 10 days after primary infection and 1 week and 5 weeks after tertiary infection were fractionated into sIg^- and sIg^+ subpopulations. In 4 separate experiments the original cell population, sIg^- cells, sIg^+ cells and a population which was reconstituted from separated sIg^- and sIg^+ cells were transferred into 4 groups of recipient rats which were infected with 1000 L_3 at the same time. Since it was previously established that 1×10^8 day 10 TDL can cause almost complete expulsion of the parasites by day 8 (see chapter 3), this dose of original and of reconstituted cells was given to the recipient rats (Table 4-2). Separated sIg^+ and sIg^- cells were transferred in the proportions in which they were recovered after fractionation (Table 4-2). Worm burdens were examined 8 days after infection and cell transfer.

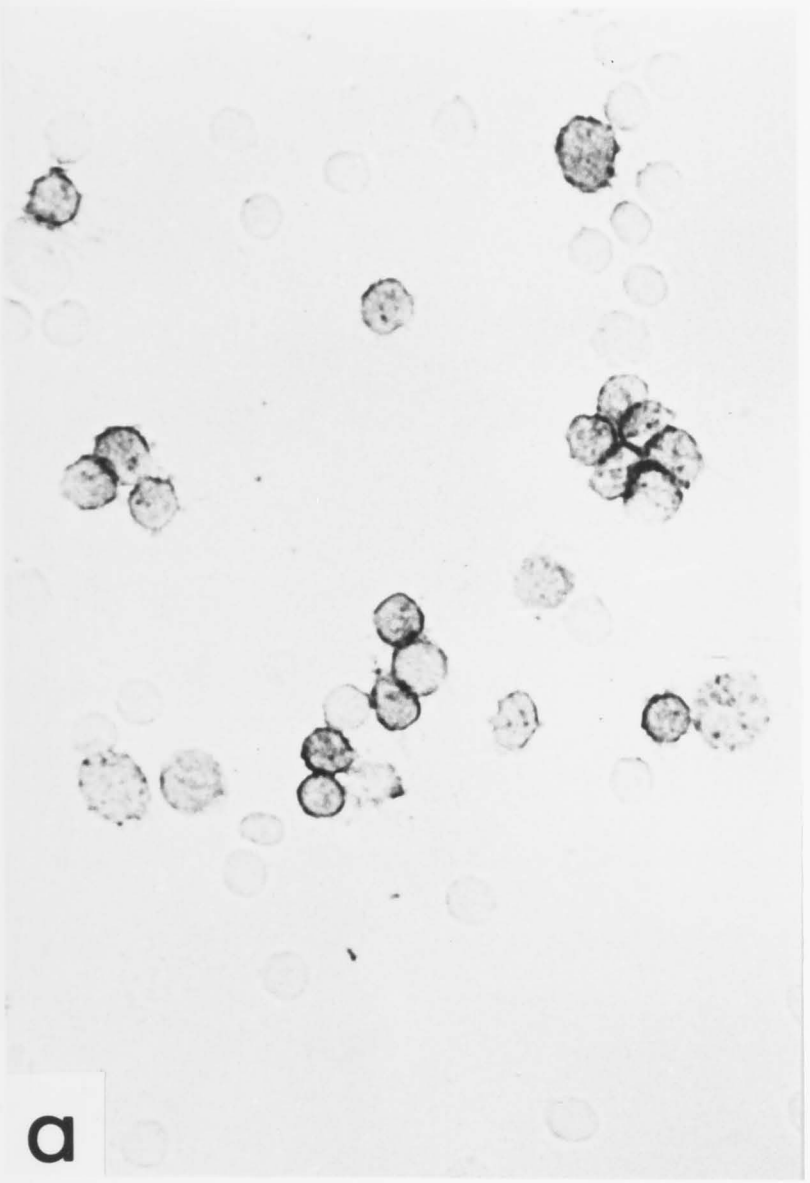
Fig. 4-1: Surface and intracellular immunoglobulins of rat TDL stained with the immunoperoxidase technique before and after fractionation.

(a) Surface Ig on rat TDL before fractionation. Cells were incubated with sheep $F(ab')_2$ anti-rat Fab-HPO. (× 800).

(b) Surface Ig on the cells of the 'upper layer' (see legend for Table 4-1) cells after fractionation. Cells were incubated with sheep $F(ab')_2$ anti-rat Fab-HPO. Note the complete absence of staining except for the endogenous peroxidase activity of an eosinophil leucocyte (× 800).

(c) Intracellular immunoglobulin in cells of the 'upper layer' after fractionation. The smear was incubated with sheep Ig anti-rat Fab-HPO (× 800).

(d) Intracellular Ig in cells of the 'lower layer' after fractionation. The SRBC ghosts have a weak endogenous peroxidase (Haemoglobin) activity. (× 800).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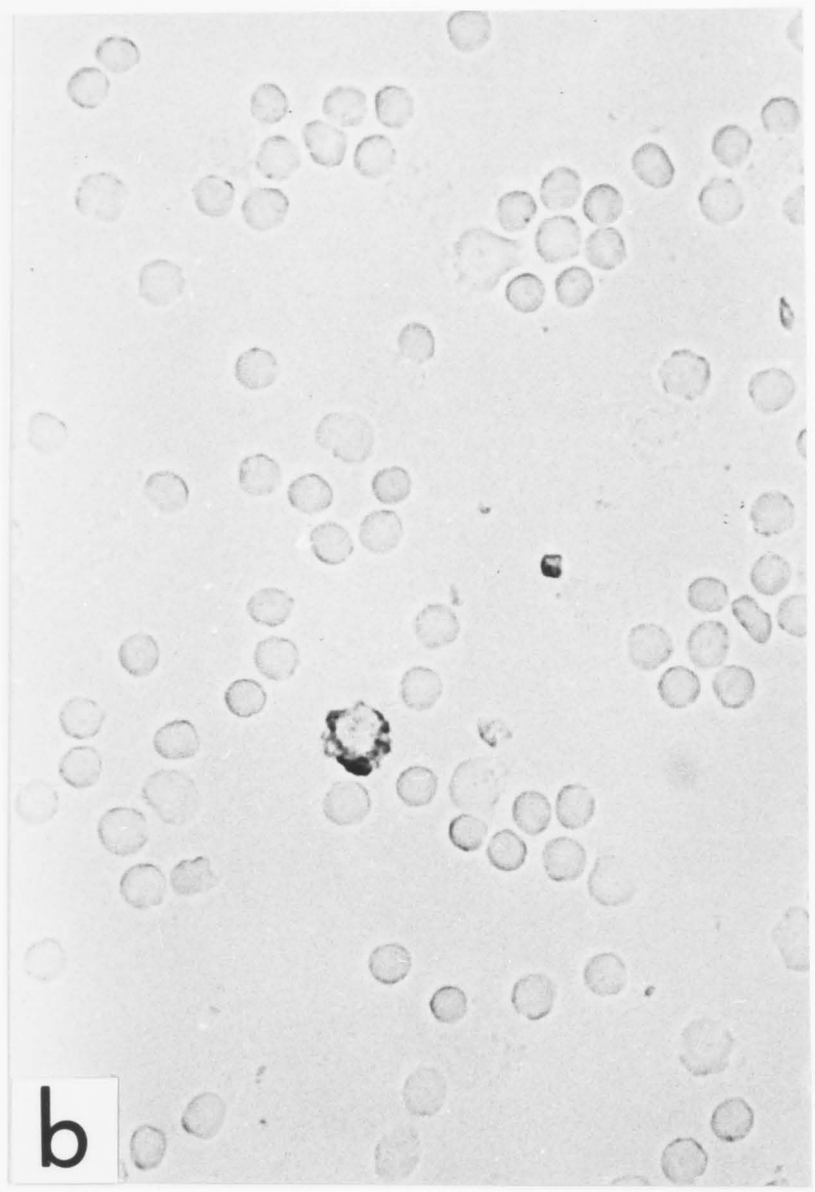
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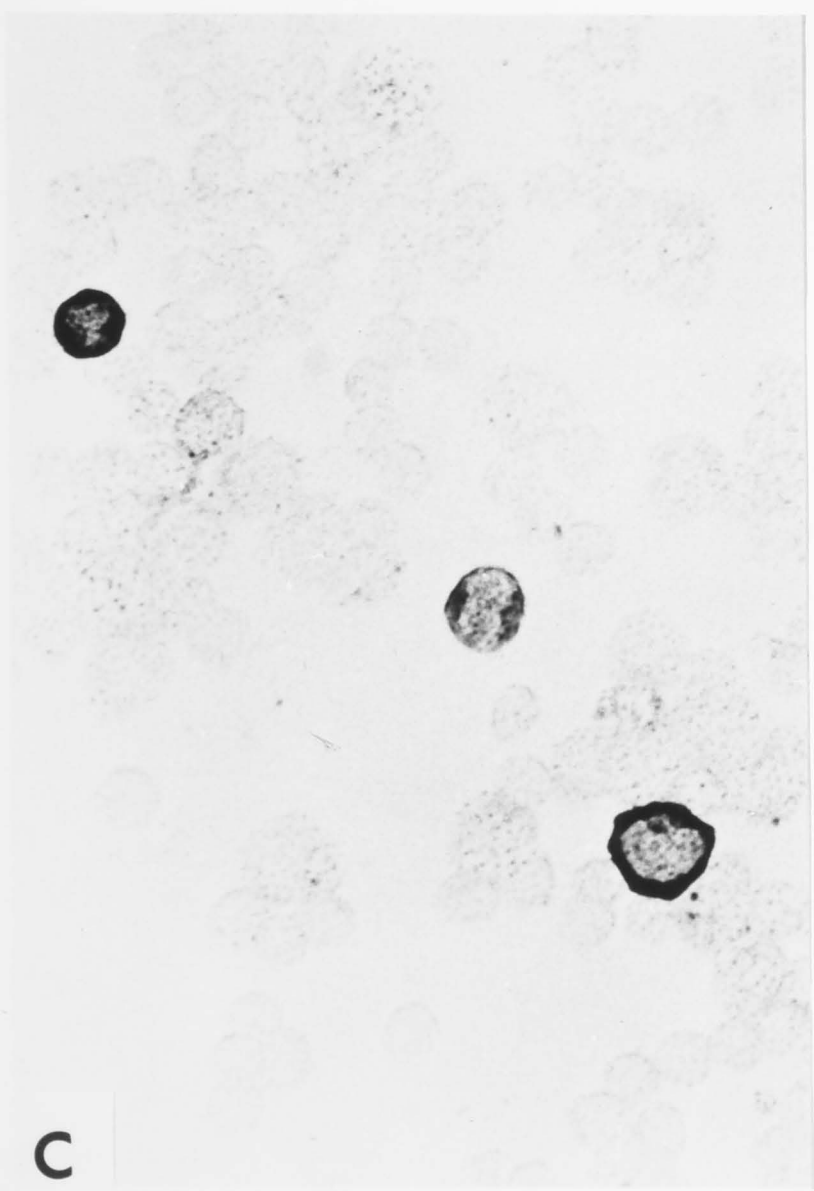
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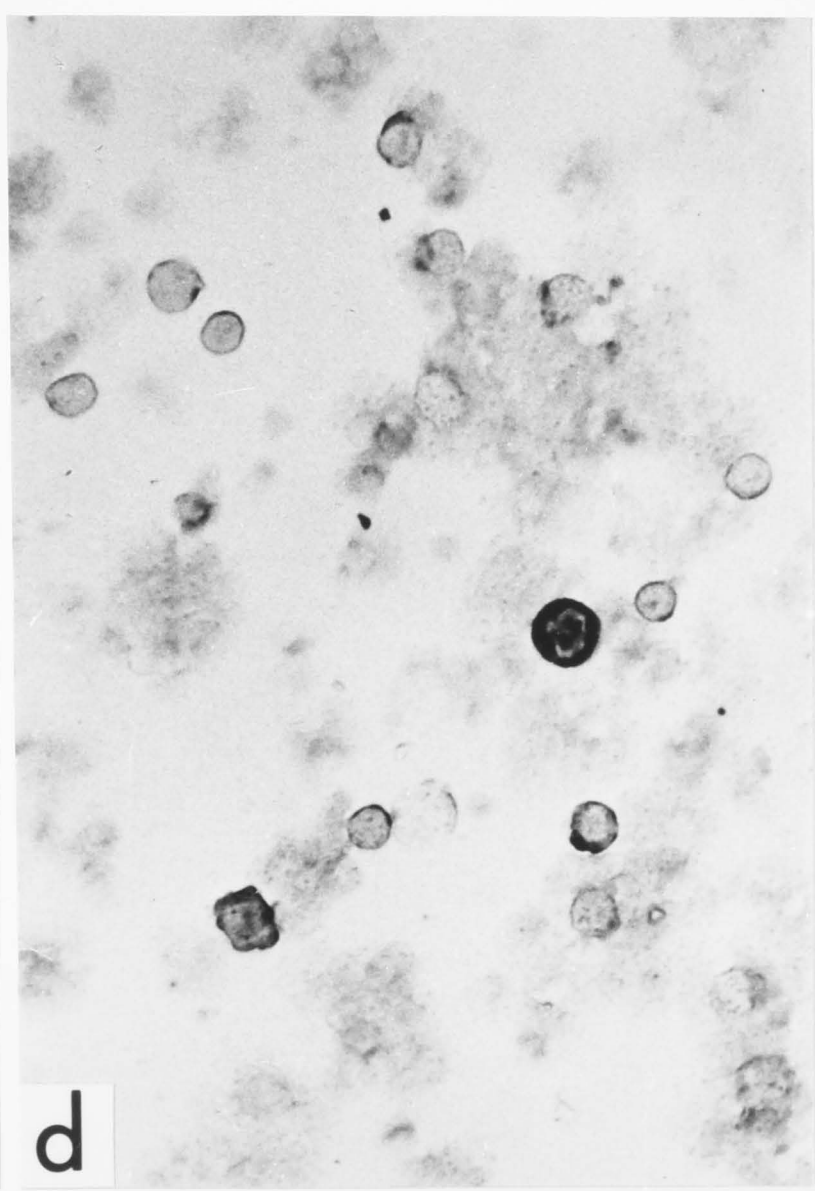
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(a) TDL obtained 10 days after primary infection

Two separate experiments were carried out to determine which subpopulation of day 10 TDL would adoptively protect infected recipients. In both experiments, the original TDL caused complete expulsion of the worms by day 8 (Table 4-2). Of the two subpopulations, only the sIg^- cells were protective but these were as effective as the original TDL population (Table 4-2). Complete protection was also observed in the recipients of the cells which had been separated and then reconstituted. In neither experiment did sIg^+ cells cause any significant reduction in worm burden when compared with that of controls given no cells (Table 4-2).

(b) Hyperimmune TDL obtained 1 week after tertiary infection

Unfractionated TDL taken 1 week after tertiary infection also brought about an early expulsion of the parasites although their efficacy was, as reported previously (chapter 3) less than that of day 10 TDL (Table 4-3). The effector cells were again predominantly in the sIg^- cell population (Table 4-3) although, slight, but highly significant ($P < 0.001$) protection was observed after transfer of the sIg^+ cell population (Table 4-3). The efficacy of the separated and reconstituted cell population was greater than that of the sIg^- cells or sIg^+ cells alone and was the same as the original cell population (Table 4-3).

(c) Hyperimmune TDL obtained 5 weeks after a tertiary infection

To determine whether an increasing proportion of the protection would, with time, become associated with sIg^+ cells, TDL were obtained 5 weeks after a tertiary infection. The fractionation procedures and the experimental protocol were the same as those in the previous experiments and the results are shown in Table 4-4.

There was a reduction in the protective effect of the unfractionated original cell population (Table 4-4) when compared with that of TDL

Table 4-2: The protective capacities of day 10 TDL after fractionation into sIg^- or sIg^+ subpopulations

(Exp. 1)

	No. of cells transferred	Worm count	% Expulsion
-	-	743 ± 140	
sIg (-)	4.5×10^7	7 ± 2	99.0 ± 0.6
sIg (+)	5.5×10^7	728 ± 27	2.0 ± 8.1
Reconstitued	4.5×10^7 sIg (-) 5.5×10^7 sIg (+)	3 ± 2	99.7 ± 0.3
Original	1×10^8	6 ± 7	99.2 ± 1.0

(Exp. 2)

-	-	652 ± 24	-
sIg (-)	6×10^7	0.5 ± 0.5	99.9 ± 0.1
sIg (+)	4×10^7	645 ± 58	1.0 ± 9.0
Reconstituted	6×10^7 sIg (-) 4×10^7 sIg (+)	0 ± 0	100 ± 0
Original	1×10^8	0.2 ± 0.2	100 ± 0

$(PvG/c \times DA)F_1$ rats were infected with 1000 L_3 and, at the same time were adoptively immunized with unfractionated cells (original), with the sIg^- and sIg^+ subpopulations, and with cells which were separated and reconstituted. A control group of 5 rats was infected but was not given cells. Worm burdens were counted 8 days after infection and cell transfer. Groups of 6 rats were given sIg^- or sIg^+ cells and groups of 5 rats were injected with the original and reconstituted cell populations. Each value represents the mean ± standard error.

obtained from donors 1 week after a tertiary infection or from donors 10 days after a primary infection. However, the effector cells were again found predominantly in the sIg^- cell population although a significant ($0.05 > p > 0.02$), but small degree of protection was also conferred by the sIg^+ cell population (Table 4-4). Both sIg^+ and sIg^- cell populations were less effective than the original unfractionated cells (Table 4-4). The separated and reconstituted cell population was, however, as effective as the original cells in conferring protection (Table 4-4).

DISCUSSION

Thoracic duct lymphocytes drained from infected donor rats of varying immunological status were fractionated into sIg^+ and sIg^- subpopulations. The abilities of unfractionated TDL, the two subpopulations and the reconstituted cells to confer protection against *N. brasiliensis* were then examined. The results clearly show that effector cells from day 10 TDL are found only in the sIg^- cell population and that they predominate in the sIg^- cell population from hyperimmune TDL. A small but significant effect is, however, found after transfer of the sIg^+ cells separated from hyperimmune TDL obtained 1 week and 5 weeks after the last challenge.

It is generally accepted that thymus-derived (T) lymphocytes have a low density of sIg whereas bursa-equivalent (B) lymphocytes express a high density of sIg (Rev. by Warner, 1974; Rev. by Marchalonis, 1975). In fact, the separation procedure used in this study has been shown previously to be particularly effective for the fractionation of rodent lymphocyte populations into functional subpopulations of T- and B-cells. For example, the separation method has shown that helper T-cells, both in rats and mice, are sIg^- (Parish and Hayward, 1974c; Parish, Kirov, Bowern and Blanden, 1974), that cytotoxic T-cells and their precursors are sIg^- (Parish *et al.*, 1974) and that T-cells which mediate delayed-type hypersensitivity responses lack sIg (Ramshaw,

Table 4-3: The protective capacities of hyperimmune TDL (1 week after tertiary infection) after fractionation into sIg^- or sIg^+ subpopulations.

	No. of cells transferred	Worm count	% Expulsion
No. cells	-	764 ± 12	-
sIg (-)	4.5×10^7	128 ± 108	83.3 ± 5.8
sIg (+)	5.5×10^7	530 ± 47	30.7 ± 6.1
Reconstituted	4.5×10^7 sIg (-) 5.5×10^7 sIg (+)	29 ± 12	96.3 ± 1.6
Original	1×10^8	20 ± 10	97.4 ± 1.2

For further details see Table 4-2.

Table 4-4: The protective capacities of hyperimmune TDL (5 weeks after tertiary infection) after fractionation into sIg^- or sIg^+ subpopulations.

	No. of cells transferred	Worm count	% Expulsion
No. cells	-	904 ± 36	-
sIg (-)	5.6×10^7	353 ± 65	61.0 ± 7.2
sIg (+)	4.4×10^7	701 ± 54	22.5 ± 6.0
Reconstituted	5.6×10^7 sIg (-) 4.4×10^7 sIg (+)	162 ± 37	82.1 ± 4.1
Original	1×10^8	146 ± 39	83.9 ± 4.3

For further details see Table 4-2.

Bretcher and Parish, 1976). In contrast, resting B-lymphocytes and splenic antibody-forming cells are sIg^+ (Parish and Hayward, 1974c; Parish *et al.*, 1974).

Since there is ample evidence that expulsion of the adult worms from the gut is, in some way, mediated by antibody (Rev. by Ogilvie and Jones, 1971, 1973; Ogilvie and Love, 1974; see also chapter 1 of this thesis) and is a thymus-dependent process (Ogilvie and Jones, 1967; Wilson *et al.*, 1967; Kelly, 1971, 1972; Jacobson and Redd, 1974, 1976; see also chapter 1), the simplestinterpretation of the present results is that a highly T-dependent antibody response is being transferred. Thus protection was very effectively transferred by the sIg^- population from day 10 TDL because this population should be rich in T-helper cells and could, therefore, facilitate the induction of a protective antibody response in the recipients.

After fractionation of hyperimmune TDL, sIg^- and to a much lesser extent, sIg^+ cells conferred protection, and in this case it seems likely that memory helper T-cells reside in the sIg^- population and that memory B-cells are being transferred in the sIg^+ population. This concept is supported by the findings of Cunningham and Sercarz (1971) where memory helper T-cells appeared shortly after antigen stimulation and contributed to a primary immune response by activating virgin B-cells, whereas development of memory AFC precursors took much longer and required a larger dose of antigen.

Recent observations by Ogilvie *et al.*, (1977) who demonstrated that transferred 'damaged' worms were expelled from irradiated (750 rad) recipients after the transfer of sIg^- cells, do not support the view that sIg^- cells are conferring T-cell help. The latter group have proposed that the sIg^- cells themselves are capable of expelling the antibody-damaged parasites. However, as Ogilvie *et al.*, (1977) have pointed out, the severe

effects of irradiation on the gut may render it unsuitable for the parasites such that even a mild immunological insult might bring about worm expulsion.

Despite the proven efficiency of the separation technique used in the present experiment (Parish and Hayward, 1974a; Parish *et al.*, 1974), not all of the Ig-synthesizing cells were removed from the sIg^- population. These cells presumably are equivalent to antibody-forming cells with a low density of sIg described by others (Paraskevas, Lee and Israels, 1970; Hämmerling and Rajewsky, 1971; Bankert, Wolf and Pressman, 1976) and may be capable of proliferating in the recipients to produce protective antibodies. Moreover, my own preliminary studies have shown that the majority of these cells synthesize IgA. In view of the findings of Pierce and Gowans (1976) that IgA-synthesizing anti-cholera toxoid antibody-forming cells appeared in thoracic duct lymph after oral boosting and that such cells tend to localize in the gut, the present results as well as those of Ogilvie *et al.*, (1977) should be interpreted with some caution until the role of specific antibody has been fully characterized.

Concomitant studies on IgE synthesis provide good supporting evidence as to the efficacy of the separation technique (chapter 6). Although no IgE antibody was detected in the sera of any of the groups given day 10 TDL there was a good response in groups given hyperimmune TDL. Moreover, when the latter were fractionated, neither the sIg^+ nor the sIg^- population alone was able to confer an IgE response whereas the reconstituted population was as effective as the original cells in this regard (chapter 6).

The complexity of the mucosal events involved worm expulsion is high-lighted by studies of the mast cells (chapter 5). Day 10 TDL were able to confer a strong IMC response and, after fractionation, it was the sIg^- but not the sIg^+ cells which brought about an increase in mast cells. In contrast, hyperimmune TDL were much less efficient at conferring the IMC

response yet it was the sIg^- population of these cells which caused a slight increase in IMC. These observations serve to emphasize the importance of examining each of the various components of the response and the caution with which the results of the worm burden kinetics must be interpreted.

CHAPTER 5

THE INTESTINAL MAST CELL RESPONSE

CHAPTER 5

THE INTESTINAL MAST CELL RESPONSE

Mast cells contain a wide range of biologically active substances. These include heparin, histamine, 5-HT, a chymotrypsin-like enzyme and SRS-A (Rev. by Csaba and Nilzén, 1974). Mast cells also have receptors for homocytotropic antibody (IgE) on their surfaces (Ishizaka and Ishizaka, 1975) and, following the binding of antigen to this antibody, the mast cells degranulate, thus releasing their content of biologically active substances (Ishizaka and Ishizaka, 1975). The mast cell-homocytotropic antibody system has, therefore, an important role in the pathogenesis of a variety of allergic conditions. However, a beneficial and protective function for the allergic response has yet to be clearly demonstrated although Lichtenstein (1972) has suggested that, teleologically, there must be one.

An attractive hypothesis, originally proposed by Urquhart *et al.* (1965), is that local anaphylaxis may cause the immune expulsion of the nematode parasite *N. brasiliensis* from the intestine of the rat. This hypothesis was supported by subsequent experiments (Barth *et al.*, 1966) which showed that heterologous sensitization and anaphylaxis promoted the effects of immune serum against the parasite. Similarly, a marked increase in mucosal permeability was observed at the time of worm expulsion during a primary infection (Jarrett, Miller, Murray, 1970; Murray *et al.*, 1971a) and the administration of monoamine-depleting or blocking drugs to *N. brasiliensis*-infected rats partially prevented the expulsion of the parasites (Urquhart *et al.*, 1965; Murray *et al.*, 1971c; Sharp and Jarrett, 1968).

Since Taliaferro and Sarles (1939) first reported the involvement of mast cells and eosinophils in the mucosal response to the parasite several studies on mast cells have been undertaken in rats infected with *N. brasiliensis*. It has been shown that intestinal mast cells (IMC) increase in number at, or about the time of worm expulsion (Wells, 1962; Jarrett, Jarrett, Miller and Urquhart, 1967; Miller, 1969; Murray *et al.*, 1971a; Miller and Jarrett, 1971). IMC are derived from cells which are indistinguishable from lymphoid blast cells and they undergo division and differentiation *in situ* (Miller, 1969; Miller, 1971a; Miller and Jarrett, 1971). They degranulate and migrate into the intestinal epithelial layer to become globule leukocytes during the final rapid phase of worm expulsion (Miller *et al.*, 1967; Murray, *et al.*, 1968; Miller, 1971b; Miller and Walshaw, 1972). Recently Mayrhofer *et al.* (1976) demonstrated that both IMC and globule leukocytes contain IgE intracellularly, and in this regard, as well as in their morphological and histochemical properties (Enerbäck, 1966a,b,d; Miller and Walshaw, 1972), they are quite distinct from connective tissue mast cells.

Little is known about the origin of the mucosal mast cells or of the stimuli which bring about their proliferation and differentiation. Nor is it certain that they are involved in worm expulsion since several workers have been unable to detect them until after the worms have been expelled (Keller, 1971; Kelly and Ogilvie, 1972).

The purpose of the present series of experiments was, therefore, to follow the IMC response in adoptively immunized rats and to re-examine, in detail, the relationship between IMC and worm expulsion. The results show that the IMC response can be transferred by adoptive immunization and that IMC may be derived from a subpopulation in the transferred immune TDL. In addition, there is a clear-cut relationship between worm expulsion and the rise in IMC numbers.

RESULTS

Strain differences

Groups of outbred Wistar and inbred DA and PvG/c rats were infected with 3000 L_3. Five rats from each group were killed at various times after infection and worm burdens were counted. Histological specimens were taken from all animals for the examination of mast cells. Both worm burden kinetics and mast cell kinetics are shown in Fig. 5-1. Furthermore, because (PvG/c × DA)F_1 rats were used for adoptive immunization, mast cell and worm burden kinetics were examined in this strain after infection with either 1000 L_3 or 4000 L_3 as described previously (chapter 1).

Both the worm burden kinetics and the mast cell responses were different in each strain of rat (Figs 5-1 and 5-2). The parasites were completely expelled by day 12 both in DA rats (Fig. 5-1) and in (PvG/c × DA)F_1 rats infected with 1000 L_3 and by day 14 in (PvG/c × DA)F_1 rats infected with 4000 L_3. On the other hand, more than 400 worms were found on day 14 in PvG/c rats and variable numbers of worms (2 ~ 460) were still present on day 16 in this strain (Fig. 5-1). The worm burden kinetics of outbred Wistar rats (Fig. 5-1) were similar to those described by Jarrett *et al.* (1968) in hooded Lister rats. The worm burden remained stable until day 11 whereafter the parasites were rapidly expelled between days 12 and 14 (Fig. 5-1). Small numbers of residual worms were observed on day 16 (Fig. 5-1).

Mast cells in the intestinal mucosa had disappeared by day 8 in all the strains examined (Figs. 5-1 and 5-2). Newly formed mast cells similar to those described previously (Miller and Jarrett, 1971; Miller, 1971a) appeared on day 10 in the DA strain, on day 12 in the (PvG/c × DA)F_1 rats infectwd with 1000 L_3 or 4000 L_3, on day 13 in the Wistar rats, but not until day 15 in the PvG/c strain (Figs. 5-1 and 5-2). The timing of the appearance of mast cells in the intestinal mucosa coincided with the

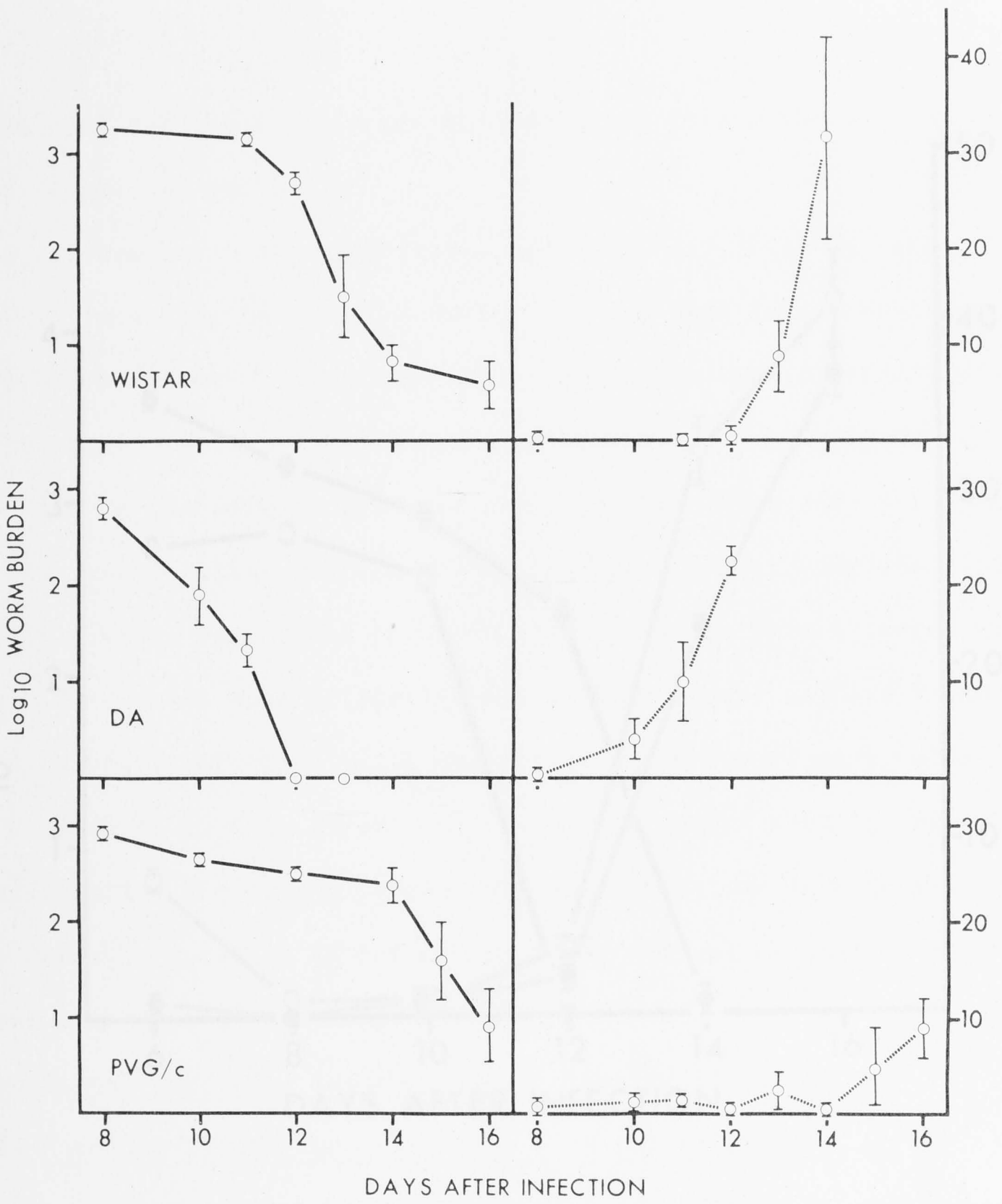


Fig. 5-1: Worm burden kinetics (o —— o) and the intestinal mast cell response (o---o) in outbred Wistar rats and in inbred DA and PvG/c rats infected with 3000 L_3. Worm burdens are recorded as $\log_{10}$ geometric mean $\pm$ standard error and the numbers of mast cells are expressed as the arithmetic mean $\pm$ standard error for groups of 5 rats.

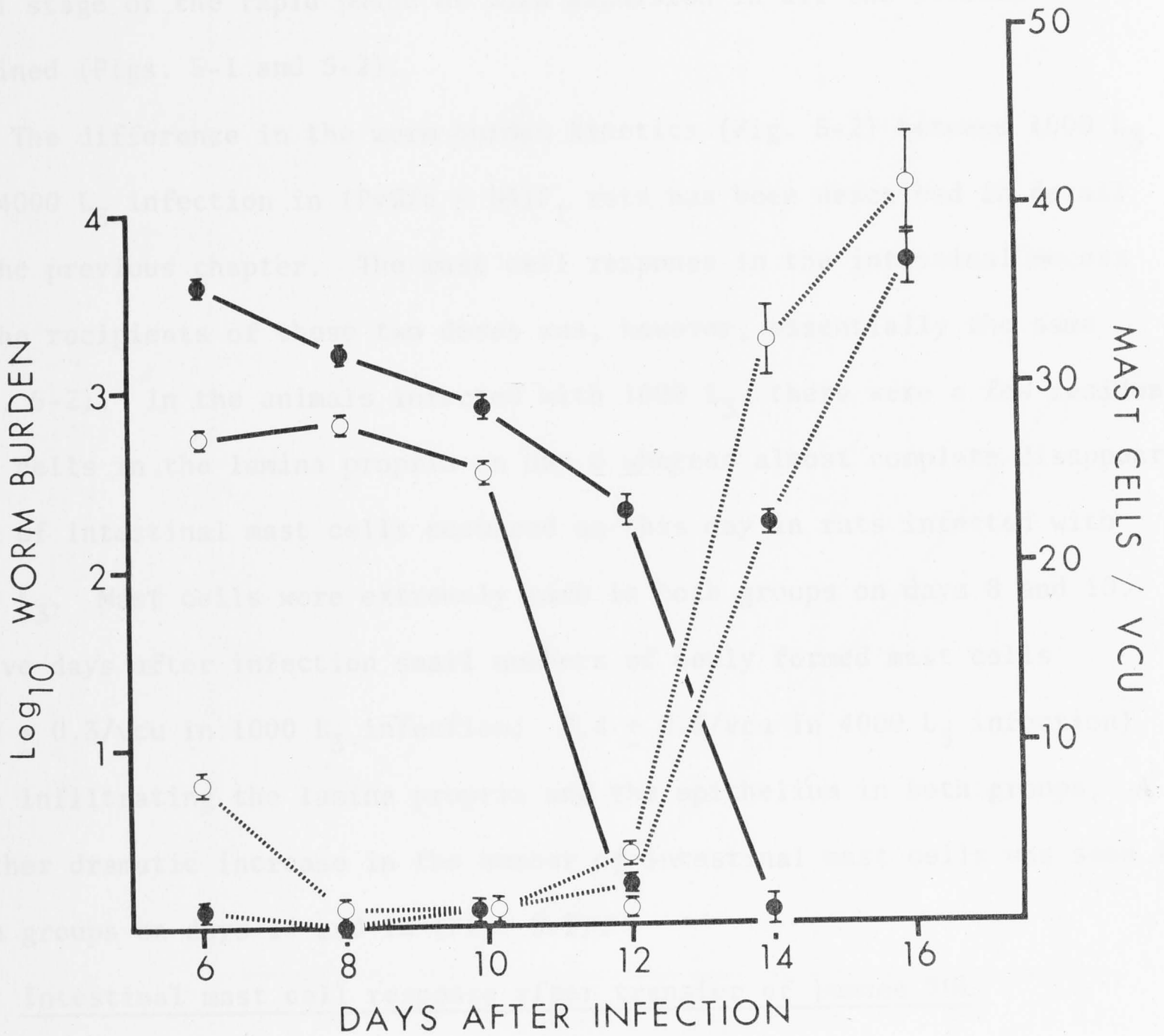


Fig. 5-2: Kinetics of intestinal mast cells (----) and the worm burdens (——) in (PvG/c × DA)F_1 rats infected with 1000 L_3(O) or with 4000 L_3 (●). The mast cell data is expressed as the mean ± SE for groups of 5 rats and the worm burdens are transformed to $\log_{10}$ of the geometric mean ± SE.

final stage of the rapid phase of worm expulsion in all the strains examined (Figs. 5-1 and 5-2).

The difference in the worm burden kinetics (Fig. 5-2) between 1000 L_3 and 4000 L_3 infection in (PvG/c × DA)F_1 rats has been described in detail in the previous chapter. The mast cell response in the intestinal mucosa of the recipients of these two doses was, however, essentially the same (Fig. 5-2). In the animals infected with 1000 L_3, there were a few residual mast cells in the lamina propria on day 6 whereas almost complete disappearance of intestinal mast cells occurred on this day in rats infected with 4000 L_3. Mast cells were extremely rare in both groups on days 8 and 10. Twelve days after infection small numbers of newly formed mast cells (4.0 ± 0.3/vcu in 1000 L_3 infection; 2.4 ± 0.5/vcu in 4000 L_3 infection) were infiltrating the lamina propria and the epithelium in both groups. A further dramatic increase in the number of intestinal mast cells was seen in both groups on days 14 and 16 (Fig. 5-2).

Intestinal mast cell response after transfer of immune TDL

(a) Kinetics of IMC response after a larval infection

Thoracic duct lymphocytes were obtained from donors 10 days after a primary infection (day 10 TDL) or 1 week after a tertiary infection. Recipient rats were injected with 1×10^8 immune TDL and were infected with 1000 L_3 at the same time. Control rats were infected but were not given cells. Details of the experimental protocol and the results for the worm burden kinetics have been given before (chapter 3). The kinetics of the intestinal mast cell response were examined between days 6 and 12 and the results are summarized in Figure 5-3. Six days after infection, a few residual mast cells were found in all groups. These residual cells disappeared almost completely by day 8 in the controls and in the rats given hyperimmune TDL. Mast cells were still absent on day 10 in the controls

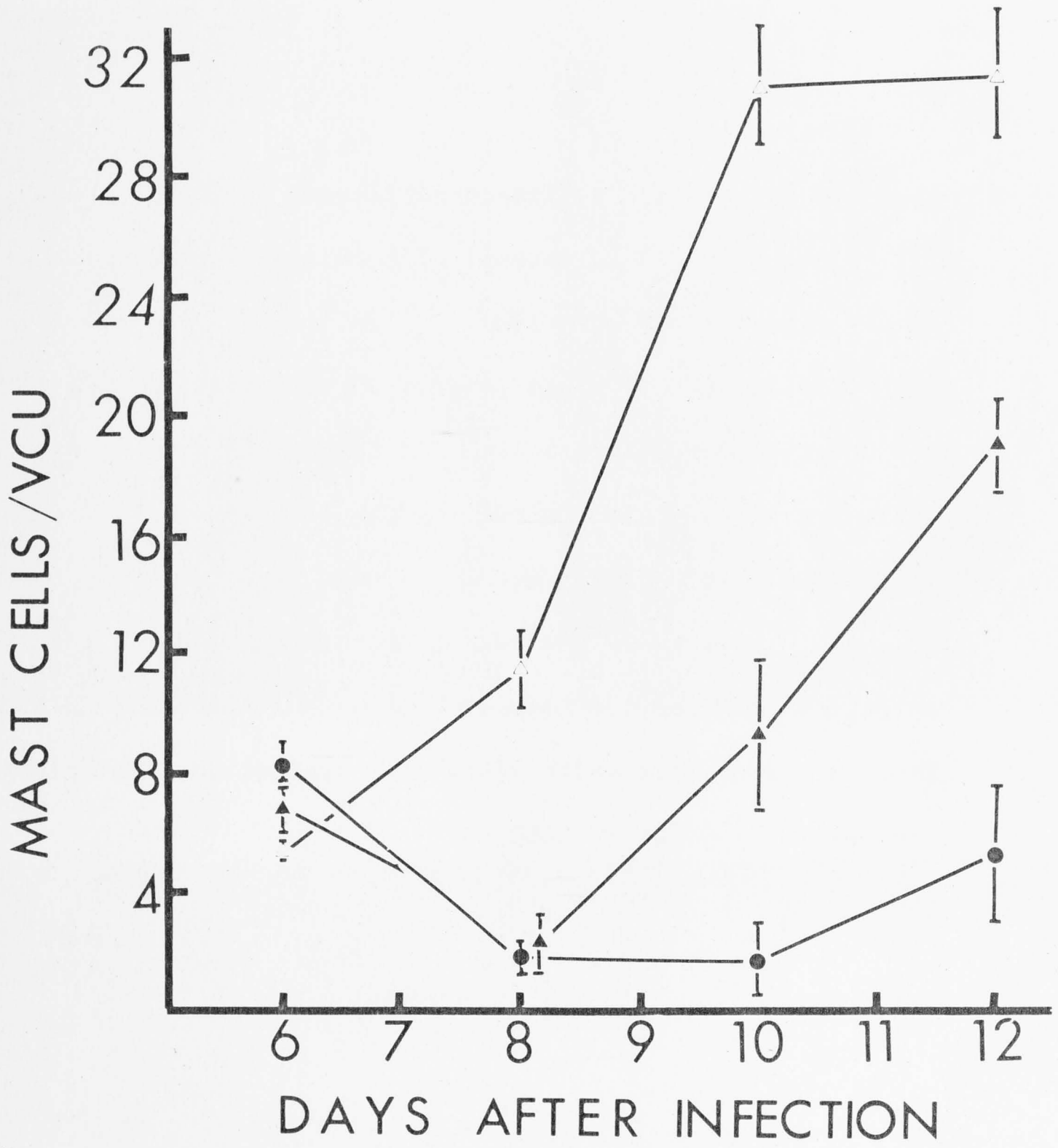


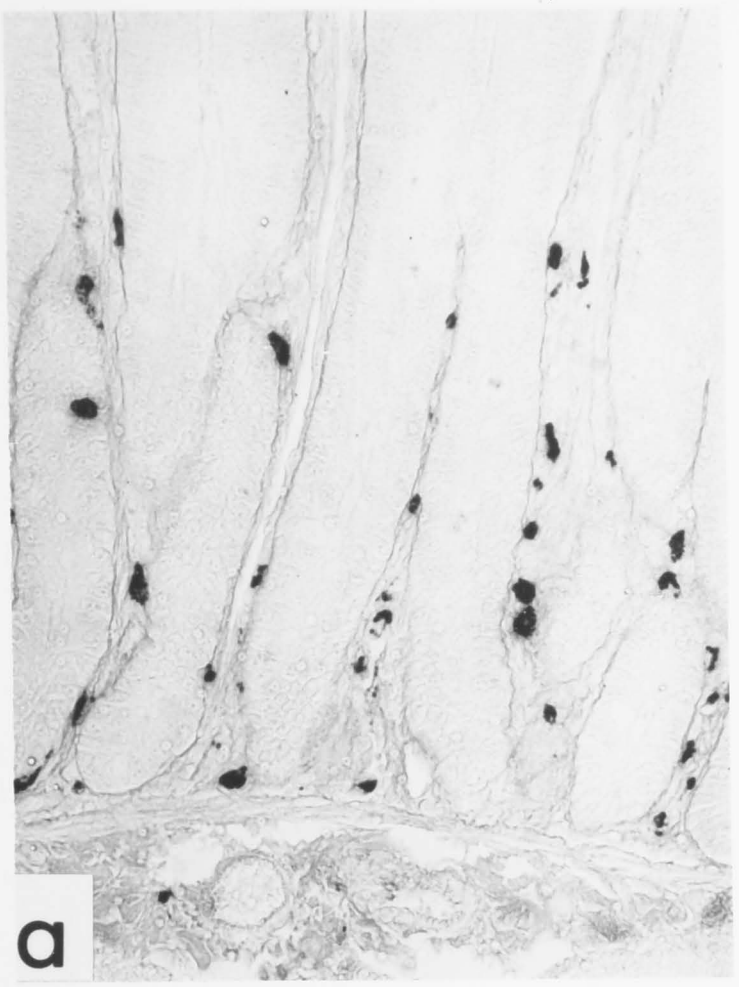
Fig. 5-3: Kinetics of the intestinal mast cell response in (PvG/c × DA)F_1 rats infected with 1000 L_3. Animals were given 1×10^8 day 10 TDL (Δ-Δ), 1×10^8 hyperimmune TDL (▲-▲) or no cells (● - ●). Each point represents the mean ± SE for a group of 5 rats.

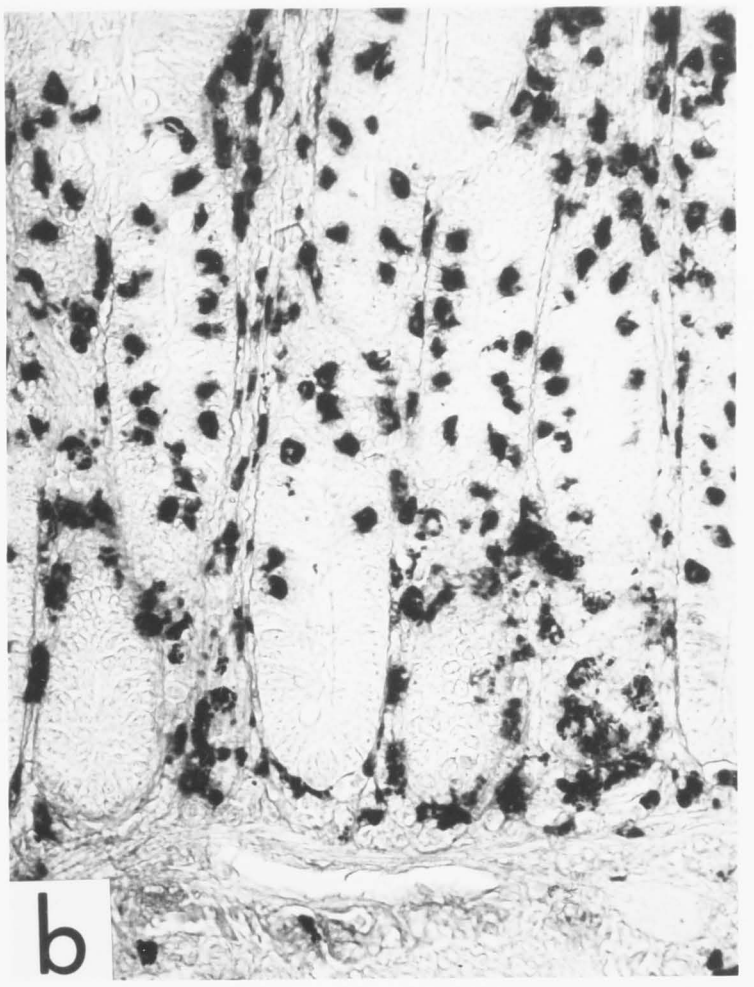
Fig. 5-4: Lightmicrographs of the intestinal mucosa of (PvG/c × DA)F_1 rats.

(a) Mast cells in the intestinal mucosa of a normal rat. Alcian blue (pH 0.3) (× 150).

(b) The intestinal mucosa 10 days after infection with 1000 L_3 and adoptive immunization with 1×10^8 day 10 TDL. Many mast cells are seen in the lamina propria and also within the epithelium of the crypt region. Alcian blue (pH 0.3) (× 150).

(c) A 1 µ section of the intestinal mucosa of a rat 8 days after infection and adoptive immunization with 1×10^8 day 10 TDL. There are many mast cells within the epithelium. Methylene blue-azure II-borax (× 2000).


a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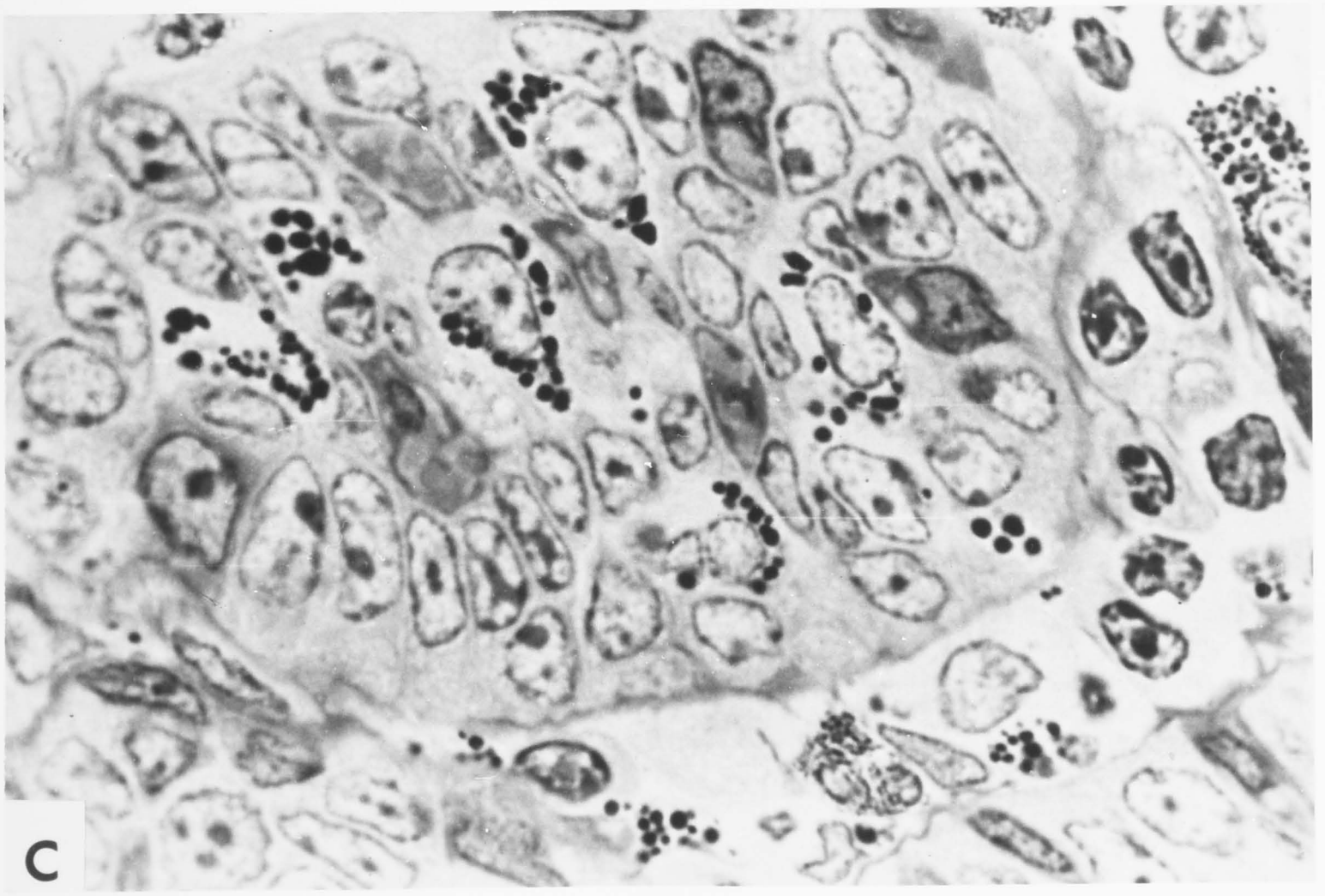
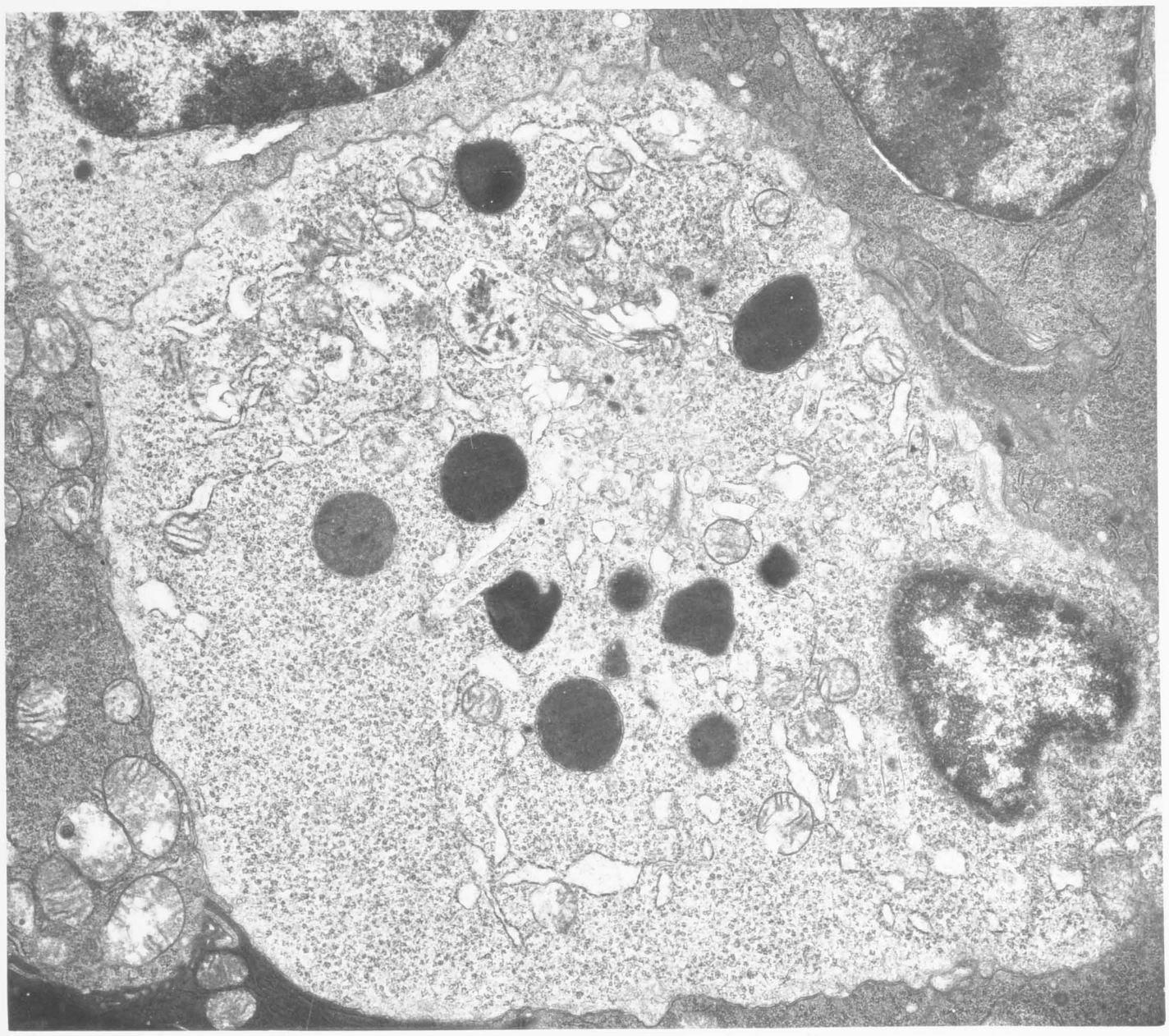

C

Fig. 5-5: Electron micrograph of an immature mast cell located within the epithelium. Aggregates of ribosomes are abundant in cytoplasm and the granules are located in the vicinity of an elaborate Golgi complex. Stained with uranyl acetate and lead citrate. (× 10,000).



(Fig. 5-3) but newly formed intestinal mast cells appeared as early as day 8 in recipients of day 10 TDL, and the peak mast cell response was reached on day 10 (Fig. 5-4b; compare with Fig. 5-4a) and remained at this level on day 12 (Fig. 5-3). Newly formed mast cells did not arise until day 10 in recipients given hyperimmune TDL and not until day 12 in control rats (Fig. 5-3). The presence of mast cells in the recipients of immune TDL was confirmed both by examination of 1 μ sections (Fig. 5-4c) and by ultra-structural observations (Fig. 5-5).

(b) Dose-response relationships after larval infection

To determine the relationship between intestinal mast cells and immune TDL, doses ranging between 1×10^7 and 4×10^8 day 10 and hyperimmune TDL were injected into recipient rats which were killed 8 days after infection and transfer of the cells (see chapter 3). Day 10 TDL were highly effective in transferring the IMC response and a linear relationship was observed in the range between 5×10^7 and 4×10^8 cells although only with doses of 1×10^8 and 4×10^8 day 10 TDL were mast cell numbers significantly increased over the infected control value. On the other hand, hyperimmune TDL, even at the highest dose, were unable to transfer the mast cell response by day 8 (Fig. 5-6).

(c) Kinetics of IMC response after implantation of adult worms

Six hundred adult worms from day 6 ('normal' worms) or from day 11 ('damaged' worms) of a primary infection with 4000 L_3 were implanted intraduodenally into recipients and, at the same time, 1×10^8 day 10 TDL were injected intravenously. Details of the experimental procedures and of the worm burden kinetics are described in chapter 3.

Figure 5-7 shows the kinetics of the worm burdens and of the IMC responses. Intestinal mast cells were completely absent on day 5 in the control animals given 'normal' worms. 'Damaged' worm implantation caused

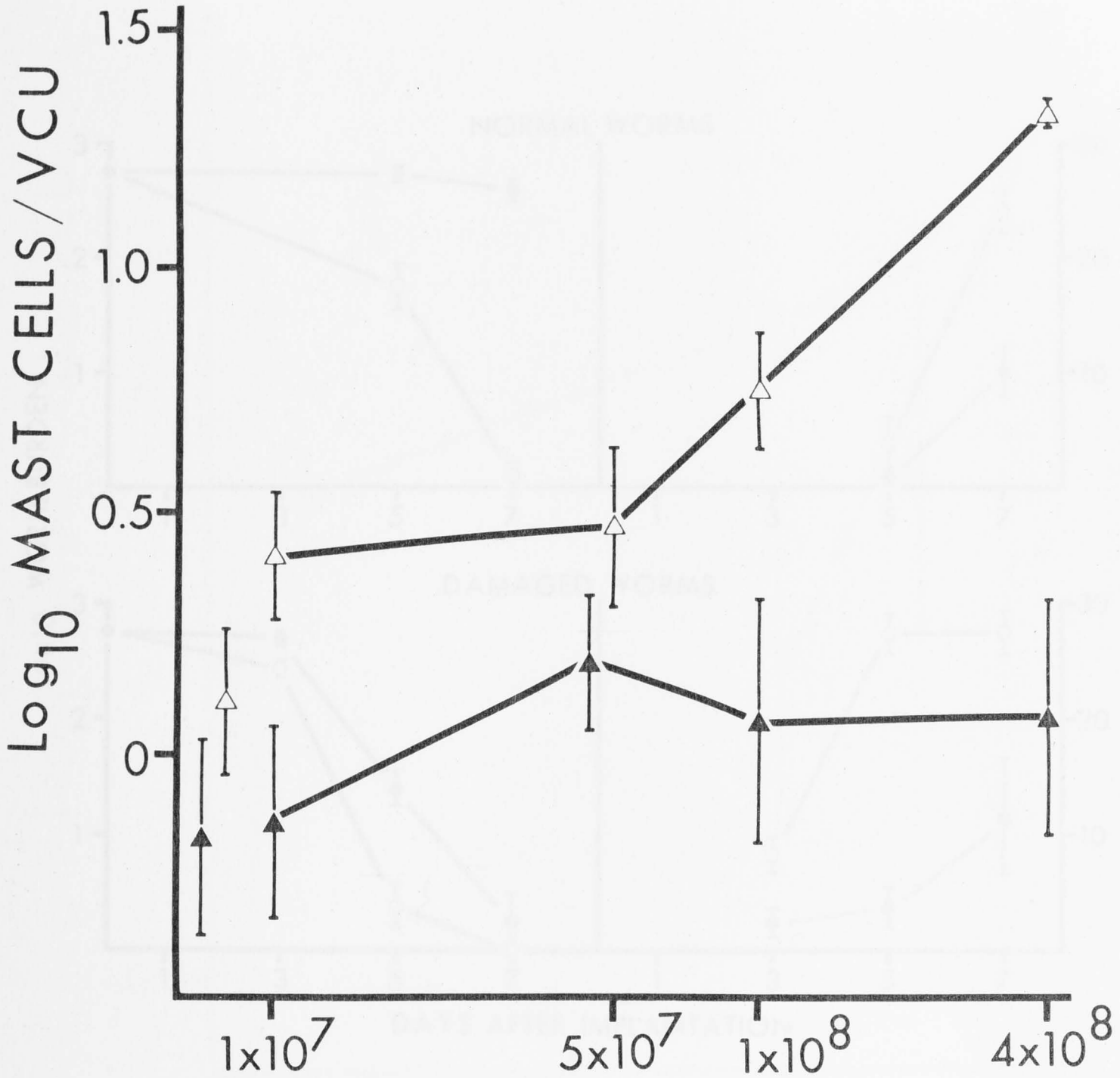


Fig. 5-6: The relationship between the intestinal mast cell response and the number of immune TDL transferred is plotted on a logarithmic scale. (PvG/c × DA)F_1 rats were infected with 1000 L_3 and, at the same time, were injected with 1×10^7, 5×10^7, 1×10^8 or 4×10^8 day 10 TDL (Δ-Δ) or with the same dose range of hyperimmune TDL (▲-▲). Intestinal mast cells were counted 8 days after infection and transfer of the cells. Control animals were infected but were not given cells. The values for the controls are plotted near the axis of the graph. Each point represents $\log_{10}$ geometric mean ± standard error for a group of 5 rats.

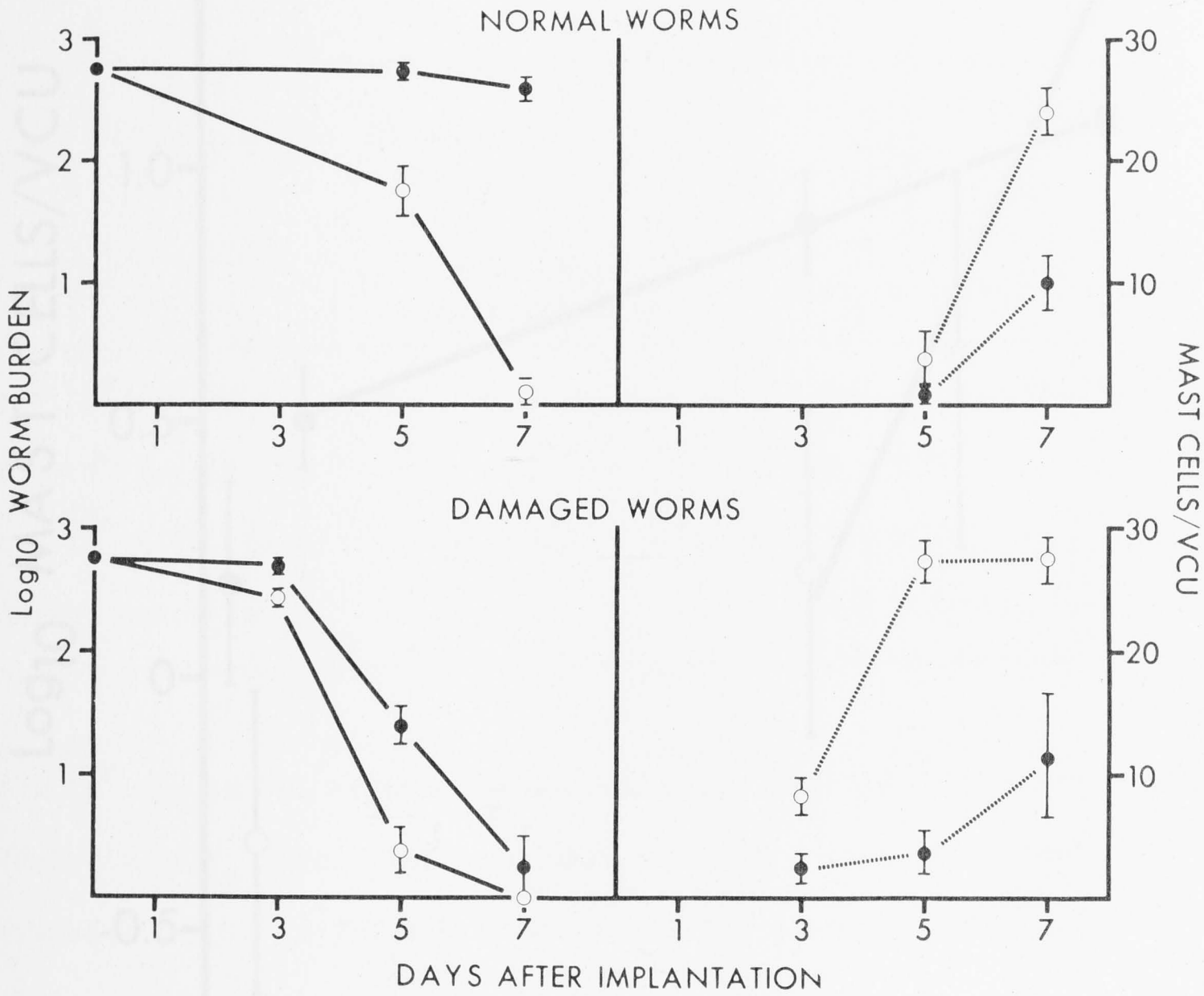


Fig. 5-7: Kinetics of the IMC response (----) and the worm burdens (——) in (PvG/c × DA)F_1 rats infected with 'normal' worms or with 'damaged' worms. Six hundred adult worms obtained on day 6 ('normal' worms) or on day 11 ('damaged' worms) of a primary infection were implanted intraduodenally into rats adoptively immunised with 1×10^8 TDL (O) and into control animals which were not given cells (●). Each point represents the mean ± standard error for a group of 5 rats, and for the worm burdens, the data is expressed as $\log_{10}$ geometric mean ± standard error.

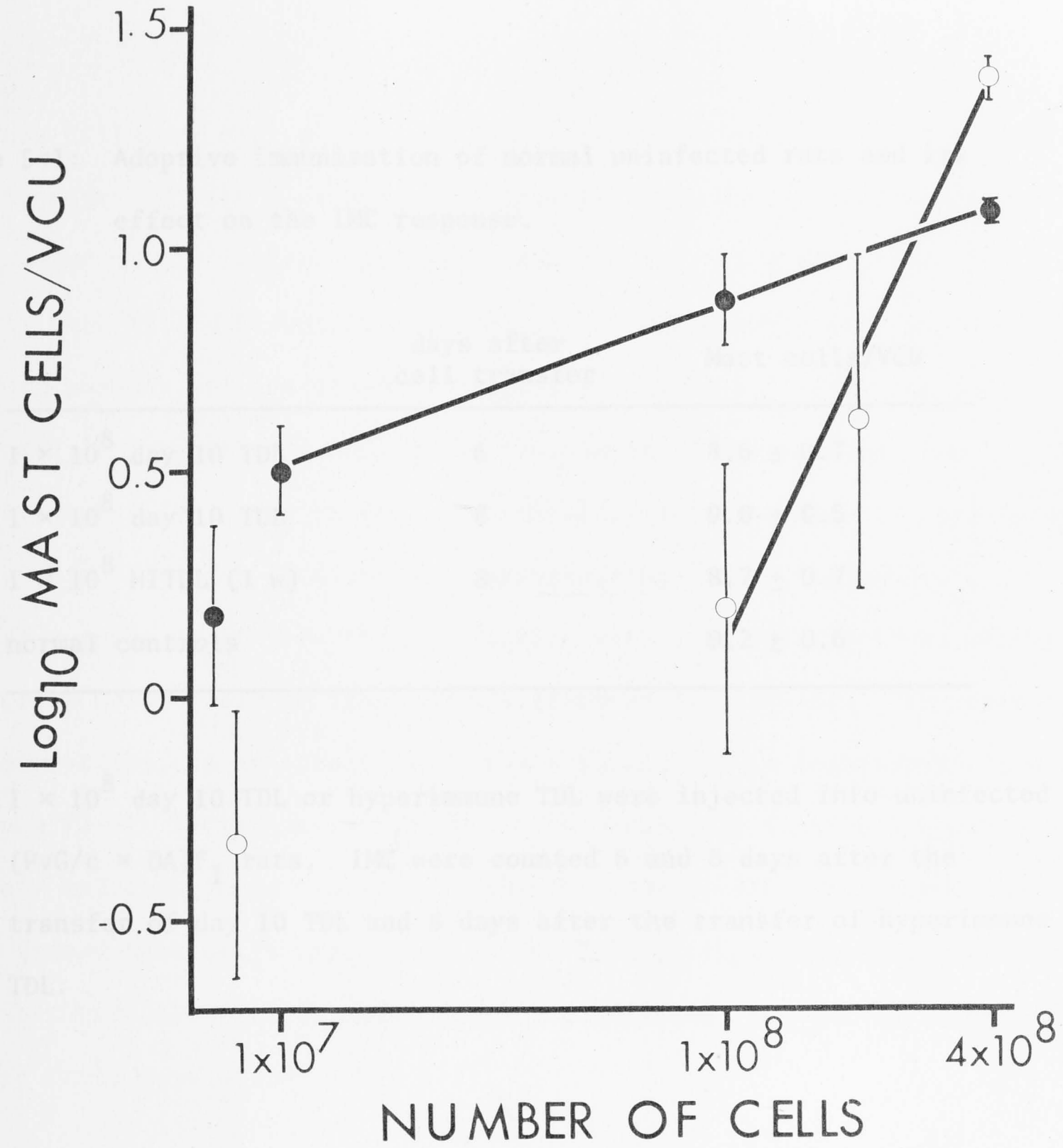


Fig. 5-8: A logarithmic plot of the relationship between the IMC response and the number of TDL transferred after implantation of adult worms. 'Normal' worms (o —— o) or 'damaged' worms (• —— •) were implanted intraduodenally into (PvG/c × DA)F_1 rats which were, at the same time, injected with different doses of day 10 TDL. The IMC response was examined 5 days after implantation of 'normal' worms or 3 days after implantation of 'damaged' worms. Control animals were given adult worms only. The control values are shown near the axis of the graph.

Table 5-1: Adoptive immunization of normal uninfected rats and its effect on the IMC response.

	days after cell transfer	Mast cells/VCU
1×10^8 day 10 TDL	6	8.6 ± 0.7
1×10^8 day 10 TDL	8	9.0 ± 0.5
1×10^8 HITDL (1 w)	8	8.7 ± 0.7
normal controls		9.2 ± 0.6

1×10^8 day 10 TDL or hyperimmune TDL were injected into uninfected (PvG/c × DA)F_1 rats. IMC were counted 6 and 8 days after the transfer of day 10 TDL and 8 days after the transfer of hyperimmune TDL.

original cells, or sIg^- or sIg^+ cells or reconstituted sIg^- and sIg^+ cells, were transferred into 4 groups of recipients which were infected with 1000 L_3 at the same time. Control rats were infected but not given cells. All animals were killed 8 days later. Details of the experimental protocol and the worm burdens are described in chapter 4. Table 5-2 shows the IMC responses in the controls and in the recipients of fractionated and unfractionated TDL. The latter, when obtained 10 days after primary infection were highly effective in adoptively transferring the IMC response. Similarly, sIg^- cells from this population were equally effective, whereas sIg^+ cells were unable to transfer the response (Table 5-2). The reconstituted population was as effective as the original TDL in transferring the IMC response (Table 5-2). Unfractionated TDL obtained 1 week or 5 weeks after tertiary infection only caused a slight increase in IMC numbers and again newly formed mast cells were found in the recipients of either sIg^- cells or reconstituted cells or the original cell population, but not in recipients of the sIg^+ cell population (Table 5-2).

DISCUSSION

The intestinal mast cell response in rats infected with *N. brasiliensis* was examined in detail and the results show that (1) it could be adoptively transferred with immune TDL: (2) the TDL which were most effective in conferring the response were those drained from donors 10 days after a primary infection; (3) there is a relationship between the numbers of IMC appearing in the mucosa and the dose of day 10 TDL transferred; (4) when TDL were fractionated, only those cells lacking sIg were able to transfer the IMC response; (5) antigenic stimulation is required for the differentiation of IMC; (6) the rise in IMC numbers is closely related to the final stage of the rapid phase of worm expulsion.

Table 5-2: The intestinal mast cell response in (PvG/c × DA)F_1 rats infected with 1000 L_3 and adoptively immunized with subpopulations of immune TDL.

	Mast Cells per Villus/Crypt Unit Mean ± SE		
	Day 10 TDL	HITDL (1w)	HITDL (5w)
-	1.8 ± 0.4[a]	1.4 ± 0.3[a]	0.5 ± 0.2[a]
sIg^-	34.9 ± 1.9[b]	5.1 ± 1.1[b]	2.5 ± 0.4[b]
sIg^+	1.4 ± 0.1[c]	1.1 ± 0.3[c]	0.7 ± 0.2[c]
sIg^- / sIg^+	43.4 ± 3.3[d]	4.3 ± 1.2[d]	3.0 ± 0.7[d]
Original	33.0 ± 1.3[e]	5.8 ± 0.9[e]	1.5 ± 0.6[e]
Statistical analysis by t-test	a vs b $P < 0.001$	a vs b $0.05 > P > 0.02$	a vs b $0.01 > P > 0.001$
	a vs c $P > 0.5$	a vs c $0.5 > P > 0.4$	a vs c $P > 0.5$
	a vs d $P < 0.001$	a vs d $0.1 > P > 0.05$	a vs d $0.02 > P > 0.01$
	a vs e $P < 0.001$	a vs e $0.01 > P > 0.001$	a vs e $0.2 > P > 0.1$

For further details see Tables 4-2, 4-3 and 4-4.

The origin of mast cells is controversial although several *in vitro* studies (Ginsburg, 1963; Ginsberg and Sachs, 1963; Ginsburg and Lagunoff, 1967; Csaba and Oláh, 1968; Combs, 1971; Ishizaka, Okudaira, Mauser and Ishizaka, 1976; Ishizaka, Adachi, Chang and Ishizaka, 1977) suggest that they may be derived from thymic and/or thymus-derived lymphocytes. Burnet (1965), from his histological observation on the thymus of NZB mice, also suggested that thymocytes could transform into mast cells. The differentiation of IMC has been studied in rats infected with *N. brasiliensis* (Miller, 1969; Miller, 1971a) and IMC were thought to be derived *in situ* from cells which were indistinguishable from lymphoid blast cells. Recently athymic (*nu/nu*) mice were reported to be unable to mount an IMC response during infection with *T. spiralis* (Ruitenberg and Elgersma, 1976) or with *N. brasiliensis* (Olson and Levy, 1976), but the IMC responses in such mice were restored with thymic grafts (Ruitenberg and Elgersma, 1976) or with transfer of thymocytes (Olson and Levy, 1976). However, the connective tissue mast cells in athymic mice were normal in their morphology and function (Keller, Hess, Riley, 1976; Ruitenberg and Elgersma, 1976; Olson and Levy, 1976). These observations, together with morphological and histochemical findings (Enerbäck, 1966a,b,d; Miller, 1969), strongly suggest that IMC are different from connective tissue mast cells and that their development is thymus-dependent.

The present results established that the IMC response is most effectively transferred by day 10 TDL and that there is a direct relationship between it and the number of day 10 TDL transferred. Furthermore, after fractionation of the TDL, the cells which caused the increase in IMC resided in the sIg^- subpopulation. These findings not only support the concept that IMC are derived from T-cells but also suggest that they might originate from the recirculating pool. However, because the recipient

animals in these experiments were not irradiated it is also possible that the transferred sIg^- cell population from immune TDL may act as 'helper' cells for the *in situ* differentiation of IMC from host lymphoid blast cells (Miller, 1969, 1971a).

Kinetic studies of IMC after the adoptive immunization of infected recipients (Fig. 5-3) and the results of the cell fractionation experiments (Table 5-2) suggest that sIg^- cells in hyperimmune TDL are also able to confer the IMC response although they are much less effective in this regard than are day 10 TDL. The reason for the different abilities of immune TDL from donors of different immunological status to confer the mast cell response is not known. One possibility is that antigenic stimulation is important because, in a primary infection, adult worms are still present in the intestine at the time of cannulation whereas, after a tertiary infection, the majority of worms have been expelled before the donor rats are cannulated (Jarrett, Jarrett and Urquhart, 1968). Alternatively, the effector cells in day 10 TDL may be activated 'helper' cells and those in hyperimmune TDL may be memory 'helper' cells which would require a lag period before they could generate sufficient effector cells. Whatever their role in the generation of the IMC response, it is clear that the effector cells require further antigenic stimulation because day 10 TDL were unable to confer IMC responses to normal recipients (Table 5-1).

Intraduodenal implantation of 'normal' or 'damaged' worms into recipients given day 10 TDL showed that both worm populations were sufficiently antigenic to generate an IMC response. The kinetic studies following adult worm implantation suggest that 'damaged' worms were as effective as 'normal' worms in stimulating an IMC response. Moreover, damaged worms apparently stimulated a significant increase of IMC even after transfer of as few as 1×10^7 day 10 TDL. Although no direct comparison can be made between these

two dose response experiments because of the difference in their timing, it would appear that the rise in IMC occurred earlier in the recipients of the damaged worms than in those rats given 'normal' worms. This raises the question as to whether 'damaged' worms release more antigen or whether 'normal' worms are able to suppress cell responses in the mucosa. The release of worm metabolites which are known to contain a mast cell degranulator (Miller, 1969) may act selectively against mast cells. The assumption being that 'normal' worms would produce more of this metabolite and thereby depress mast cell differentiation.

The relationship of the IMC response to worm expulsion has been argued at length and evidence for and against any temporal relationship between these events has been extensively reviewed (Jarrett and Urquhart, 1971; Ogilvie and Jones, 1971, 1973; Murray, 1972; Ogilvie and Love, 1974; Waksman and Ozer, 1976). In the present experiments, increased numbers of IMC were invariably observed during the final stages of worm expulsion. Furthermore, day 10 TDL which were the most efficient in transferring the IMC response, were also the most effective in conferring protection (chapter 3). Similarly, the effector cells both for the IMC response and for worm expulsion were found in the sIg^- subpopulation from day 10 TDL. These findings would suggest that IMC may, in some way, be involved in worm expulsion. However, a recent study by Ogilvie *et al.* (1977) showed that sIg^- cells fractionated from TDL obtained 13 days after a primary infection expelled 'damaged' worms from heavily irradiated (750 rad) rats in the absence of IMC. This finding weakens the argument for, but does not necessarily preclude, a role for IMC in the protective mechanism because, as Ogilvie *et al.* (1977) pointed out, worm survival may be different in normal and irradiated animals as a result of the intestinal damage brought about by irradiation.

In contrast with the IMC response which was transferred by day 10 TDL, the IgE response could only be adoptively transferred by hyperimmune TDL and, after subfractionation, required the presence of both sIg^- and sIg^+ subpopulations (chapter 6). This failure to relate the IgE response to the IMC response and worm expulsion reduces but does not rule out a role for local anaphylaxis in worm expulsion (Urquhart *et al.* 1965). IgE-synthesising cells were found on day 10 of a primary infection (Mayrhofer *et al.*, 1976) and, as mentioned previously, peritoneal mast cells are sensitised on day 10 (Wilson and Bloch, 1968), suggesting that mast cells could be saturated with IgE before significant levels of this immunoglobulin class are detected in the serum.

Increased numbers of IMC are present even 1 month after a primary infection (Miller, 1969) and the IMC response occur much more rapidly and in greater magnitude after secondary challenge (Taliaferro and Sarles, 1939; Whur, 1966). It could be, therefore, that IgE and mast cells are involved in protection against secondary infections. The correlation between the IMC response and the circulating IgE antibody titre (Jarrett, Haig and Bazin, 1976; Ishizaka, Urban and Ishizaka, 1976) after secondary infection with *N. brasiliensis* would support this concept.

CHAPTER 6

THE IgE RESPONSE AFTER INFECTION WITH *N. BRASILIENSIS*

CHAPTER 6

THE IgE RESPONSE AFTER INFECTION WITH *N. BRASILIENSIS*

Specific IgE antibody responses are frequently associated with parasitic infections both in man and in experimental animals (Bloch, 1967; Ogilvie and Jones, 1969). *Nippostrongylus brasiliensis* infection in rats, which has been extensively used to investigate mechanisms of IgE production (Rev. by Ishizaka, 1976), elicits a specific IgE antibody response against worm extracts (Ogilvie, 1964, 1967; Wilson and Bloch, 1968), and also potentiates the IgE antibody response against unrelated antigens (Orr and Blair, 1969; Jarrett, 1972; Jarrett and Steward, 1972; Bloch, Ohman, Waltin and Cygan, 1973; Carson, Metzger and Bloch, 1975). Furthermore, a significant increase in the total level of IgE is observed in the sera of *N. brasiliensis* infected rats (Jarrett and Bazin, 1974; Carson *et al.*, 1975, Ishizaka *et al.*, 1976; Jarrett *et al.*, 1976).

IgE-forming cells have been observed primarily in the respiratory and gastrointestinal mucosae and in the regional lymph nodes in primates (Tada and Ishizaka, 1970). Such cells are also found in the mesenteric lymph nodes of *N. brasiliensis* infected rats (Ishizaka *et al.*, 1976; Mayrhofer *et al.*, 1976).

Evidence of cell collaboration in the generation of IgE antibody responses has largely been gathered by using a hapten-carrier system and/or adoptive immunization experiments in mice (Rev. by Ishizaka, 1976), although Jarrett and Ferguson (1974) were also able to demonstrate a T-cell requirement for IgE antibody production in rats.

In the present study, the adoptive transfer of the IgE antibody response was examined during infection with *N. brasiliensis*. The results show that IgE 'memory' antibody responses can be adoptively transferred

by TDL obtained from hyperimmune donors, but not by TDL from donors harbouring a primary infection. Fractionation of TDL into subpopulation showed that cells bearing and lacking surface immunoglobulin (sIg^+ and sIg^- cells respectively) are both required to provoke a detectable IgE antibody response. Since protection against *N. brasiliensis* can also be adoptively transferred by immune TDL (chapter 3, Ogilvie *et al.* 1977) the relationship between worm expulsion and the IgE antibody response will be discussed.

RESULTS

Kinetics of the IgE response during primary infection

Two groups of rats were infected with 1000 or 4000 L_3, and the kinetics of the worm burdens and the PCA activities in their sera were examined. Five rats from each group were killed on alternate days from days 6 to 16 after infection. Specific IgE antibody was not detected in the circulation until 16 days after a 4000 L_3 infection. At this time, 3 out of 5 rats had PCA activity in their sera (2^0, 2^0, 2^3, -, -). No detectable PCA activity was found in the sera from animals infected with 1000 L_3 even 16 days after infection. These results are similar to the findings of other workers who have examined primary infections of this parasite (Jarrett *et al.*, 1976; Ishizaka *et al.*, 1976).

The IgE response after adoptive immunization with day 10 TDL.

Since it has been established that adoptive immunization with day 10 TDL caused rapid expulsion of the parasites (chapter 3) the PCA activity in sera from these adoptively immunized rats was measured to determine the relationship of IgE to worm expulsion. In none of the experiments where day 10 TDL were transferred was there a detectable IgE antibody response. Some of these negative results are shown in Tables 6-1 and 6-2 which demonstrate that worm expulsion occurred in the absence of circulating IgE antibody.

Dose-response relationships in the generation of IgE response

Although the IgE response could not be transferred adoptively with

day 10 TDL, dose-response experiments using hyperimmune TDL revealed that a 'memory' type IgE response agianst worm antigen could be transferred by circulating lymphocytes. In this experiment, various doses (1×10^7, 5×10^7, 1×10^8, 4×10^8) of immune TDL obtained from donors 1 week after tertiary infection or 10 days after primary infection were transferred intravenously into recipients which were infected with 1000 L_3 at the same time. Infected animals not given cells and non-infected animals given 1×10^8 immune TDL served as controls. Recipient rats were killed 8 days after infection and cell transfer and the results of these experiments are shown in Table 6-1.

As few as 1×10^7 TDL obtained 1 week after tertiary infection were able to transfer the IgE response, the magnitude of which was dependent on the number of cells transferred (Table 6-1). When 1×10^8 hyperimmune TDL were transferred into normal animals, no IgE response occurred. This suggests that the adoptive transfer of the IgE response requires further antigenic stimulation. As mentioned previously, no PCA activity was observed in any of the infected recipients of day 10 TDL.

Requirement for cell collaboration

In chapter 4 it was established that the immune expulsion of the parasite can, to a large extent, be transferred by sIg^- cell populations from immune TDL. However, Kishimoto and Ishizaka (1972) demonstrated that memory cells for IgE responses were removed by passage through anti-Fab coated Sepharose columns. More recently, using *N. brasiliensis* infection in rats, Urban, Ishizaka and Ishizaka (1977) suggested that selective proliferation of IgE-bearing cell occurs without T cells whereas the differentiation of IgE-bearing cells to IgE forming cells is highly dependent on T cells. In view of these findings, it was of interest to determine which cell types could adoptively transfer the IgE response and to examine the relationship of the latter to the expulsion of the parasites. The details of the experimental protocol

Table 6-1: PCA activity in the sera of (PvG/c × DA)F_1 rats infects with 1000 L_3 and adoptively immunized with varying doses of hyperimmune or day 10 TDL.

	Hyperimmune TDL (1W)						Day 10 TDL					
No. of cells	Worm Count ± S.E.	PCA titre (individual values)					Worm Count ± S.E.	PCA titre (individual values)				
-	587 ± 41	(-)	(-)	(-)	(-)	(-)	771 ± 29	(-)	(-)	(-)	(-)	(-)
1×10^7	536 ± 12	(-)	2^1	(-)	2^0	2^1	558 ± 35	(-)	(-)	(-)	(-)	(-)
5×10^7	269 ± 25	2^1	2^1	2^1	2^2	2^2	216 ± 31	(-)	(-)	(-)	(-)	(-)
1×10^8	135 ± 14	2^1	2^2	2^2	2^2	2^1	120 ± 66	(-)	(-)	(-)	(-)	(-)
4×10^8	53 ± 13	2^3	2^3	2^3	2^3	2^2	2 ± 1	(-)	(-)	(-)	(-)	(-)
1×10^8 into normal rats	-	(-)	(-)	(-)	(-)	(-)	-	(-)	(-)	(-)	(-)	(-)

PCA activities are expressed as reciprocals of serial two-fold dilutions. Infected controls were given no cells (Top line). Uninfected controls were given 1×10^8 hyperimmune or day 10 TDL and the PCA activity of their sera was tested 8 days later (Bottom line). Worm burdens in the different groups are also recorded in order to demonstrate the protective activity of the transferred cells.

have been reported in chapter 4. Briefly, unfractionated cells, sIg^+ cells, sIg^- cells and cells which were separated and reconstituted were transferred into recipients at the time of infection with 1000 L_3. Eight days later the animals were killed to determine their worm burdens and the PCA activity in their sera. The results are summarized in Table 6-2.

The IgE antibody response against the parasite was adoptively transferred with hyperimmune TDL but, as described before, not with day 10 TDL obtained from donors harbouring a primary infection. TDL obtained 5 weeks after the final challenge were more effective than those from rats cannulated 1 week after the final challenge. When hyperimmune TDL were fractionated into sIg^- cells and sIg^+ cells, neither fraction was able to transfer the IgE antibody response to the recipients. However, reconstitution of the TDL with sIg^+ and sIg^- cells restored their ability to adoptively transfer the IgE antibody response (Table 6-2).

DISCUSSION

The results clearly show that 'memory-type' IgE antibody responses against *N. brasiliensis* worm antigen can be adoptively transferred by hyperimmune TDL. The magnitude of the IgE antibody responses in the recipients is directly related to the number of cells transferred. When hyperimmune TDL obtained 1 week after tertiary infection were transferred into normal rats, they failed to produce an IgE antibody response. This suggests that the development of IgE responses in the recipients requires further antigenic stimulation. Moreover, when hyperimmune TDL were fractionated into sIg^+ and sIg^- subpopulations, neither subpopulation was able to transfer the specific IgE response. The reconstituted population was, however, able to confer IgE antibody responses as effectively as the unfractionated hyperimmune TDL.

These observations strongly suggest that (a) memory cells for the IgE response reside in the circulating lymphocyte pool from hyperimmune animals;

Table 6-2: PCA activity in the serum of (PvG/c × DA)F_1 rats 8 days after infection with 1000 L_3 and adoptive immunization with fractionated immune TDL.

	Day 10 TDL		Hyperimmune TDL (1W)		Hyperimmune TDL (5W)	
	Worm Count S.E.	PCA titre (individual values)	Worm Count S.E.	PCA titre (individual values)	Worm Count S.E.	PCA titre (Individal Values)
	652 ± 24	(-) (-) (-) (-) (-)	764 ± 12	(-) (-) (-) (-) (-)	904 ± 36	(-) (-) (-) (-) (-)
SIg (-)	0.5 ± 0.5	(-) (-) (-) (-) (-) (-)	128 ± 108	(-) (-) (-) (-) (-) (-)	353 ± 65	(-) (-) (-) (-) (-)
SIg (+)	645 ± 58	(-) (-) (-) (-) (-) (-)	530 ± 47	(-) (-) (-) (-) (-) (-)	701 ± 54	(-) (-) (-) (-) 2^1
SIg (-) / SIG (+)	0 ± 0	(-) (-) (-) (-) (-)	29 ± 12	2^2 2^1 2^2 2^2 2^2	162 ± 37	2^4 2^3 2^5 2^4 2^4
Original	0.2 ± 0.2	(-) (-) (-) (-) (-)	20 ± 10	2^3 2^3 2^2 2^2 2^3	146 ± 39	2^3 2^5 2^6 2^4 2^6

The worm burdens in the different group are also shown. Details of the numbers of cells transferred are in Tables 4-2, 4-3 and 4-4. Worm burdens are expressed as the mean ± standard error. PCA activities are recorded in individual rats as the reciprocals of serial two-fold dilutions.

(b) specific IgE antibody in adoptively immunised rats is produced by cells of donor origin; (c) production of IgE antibody is triggered by re-exposure to antigen; (d) collaboration between sIg^- and sIg^+ cell subpopulations, presumably T- and B-lymphocytes, is required to produce specific IgE antibody.

Tada and Ishizaka (1970) reported that IgE-forming cells were mainly localised in respiratory and gastrointestinal mucosae and in the lymph nodes regional to these areas, but they were unable to demonstrate IgE-forming cells in human periperhal blood. Kishimoto and Ishizaka (1972) found that memory cells for the IgE response were present in the mesenteric lymph nodes of rabbits immunized with DNP-Ascaris and that these cells were distinct from memory cells for IgG and/or IgM antibodies. The present results strongly suggest that circulating lymphocytes can also carry memory for the IgE response.

The memory cells for an IgG response bear IgG on their surface and are present in a subpopulation amounting to less than 10% of all B cells in thoracic duct lymph from rats primed with DNP-BGG (Mason and Gowans, 1976). This finding, together with the observation by Kishimoto and Ishizaka (1972) that memory B-cells for IgE responses are distinct from those for IgG/IgM antibodies with respect to their surface immunoglobulin, would suggest that the circulating memory B-cells for IgE responses bear ε-chain determinants on their surfaces.

Although IgE-forming cells are predominantly found in the respiratory and gastrointestinal mucosae and in the regional lymph nodes in primates (Ishizaka, Ishizaka, Tada and Newcomb, 1969; Tada and Ishizaka, 1970; Brown, *et al.*, 1975), Mayrhofer *et al.* (1976) have shown that most of the IgE-containing cells in the lamina propria of the rat intestine were mast cells and not plasma cells. In this regard, it is possible that since

day 10 TDL conferred a strong intestinal mast cell response (chapter 5), the failure to adoptively transfer a serum IgE response with this source of cells might be because specific IgE antibody is absorbed by IMC. Conversely, since hyperimmune TDL caused only a weak mast cell response, any IgE produced in excess of the capacity of mast cells would be detected in the serum.

Accumulated evidence suggests that collaboration of T and B lymphocytes is required for IgE production (Rev. by Ishizaka, 1976; Jarrett and Ferguson, 1974). The present findings where both sIg^+ and sIg^- subpopulations were required to adoptively transfer the specific IgE response strongly support this concept.

Finally, despite the fact that the adoptive transfer of the IgE antibody response required further antigenic stimulation, we were unable to demonstrate any protective role for circulating IgE against the parasite itself. Thus, worm expulsion can be effected entirely by sIg^- cells fractionated from day 10 TDL, partially by sIg^- cells from hyperimmune TDL and to a very minor extent by sIg^+ cells from hyperimmune TDL. None of these subpopulations transferred a detectable IgE response. Indeed, there appears no particular relationship between the ability of a given cell population to effect worm expulsion and its ability to transfer the IgE response. These observations suggest that circulating IgE antibody itself has no significant role in worm expulsion. However, it is possible to speculate that small amounts of locally produced, non-circulating IgE antibody might still be involved in the expulsive mechanism. Fragments of IgE have been found in human intestinal fluids (Waldman, Virchow and Rowe, 1973; Brown and Lee, 1976) and Mayrhofer *et al.* (1976) found that IgE-forming cells in the mesenteric lymph node of rats 10 days after primary infection with *N. brasiliensis*. Furthermore, Wilson and Bloch (1968) have

shown that peritoneal mast cells are sensitized as early as day 10 after infection. Since the number of intestinal mast cells increases during the final stage of worm expulsion (chapter 5) and that this mast cell response can be adoptively transferred by sIg^- cells from day 10 TDL, it is possible that the interaction of IgE with intestinal mast cells might still be important in the final phase of worm expulsion.

CHAPTER 7

MUCOSAL PERMEABILITY DURING INFECTION

CHAPTER 7

MUCOSAL PERMEABILITY DURING INFECTION

Although IgE is produced primarily in tissues adjacent to mucous surfaces (Tada and Ishizaka, 1970) probably as a result of continuous antigenic challenge across the mucosa (Rev. by Ishizaka, 1976), the protective function, if any, of this immunoglobulin class is poorly understood. The early experiments of Stewart (Rev. by Stewart, 1959) and, subsequently, those of Urquhart *et al.* (1965) had suggested that local anaphylaxis could render the gastrointestinal mucosa unsuitable for the survival of helminth parasites. The role of local anaphylaxis was extended when the proposal that it might enhance the passage of specific antibody across mucous membranes (Barth *et al.*, 1966; Ishizaka, *et al.* 1969) was given strong support by experiments which demonstrated that the induction of a heterologous intestinal anaphylactic shock enhanced the effects of passively transferred antibodies against *N. brasiliensis* (Barth *et al.*, 1966). Additional evidence for this proposal was the finding that IMC in *N. brasiliensis*-infected rats increased in number and degranulated at the time of worm expulsion (Miller, 1969; Miller and Jarrett, 1971; Murray *et al.*, 1971a; Miller, 1971a,b; Miller and Walshaw, 1972).

Murray *et al.* (1971a) reported that the rapid phase of worm expulsion was accompanied by a significant increase in the passage of ^{131}I-polyvinyl pyrrolidone(PVP) into the gut lumen and they proposed (Jarrett *et al.*, 1970; Murray *et al.*, 1971a) that the interaction of worm allergen with IgE bound to the IMC caused increased mucosal permeability. Ultrastructural evidence of mucosal damage (Miller, 1969; Murray *et al.*, 1969; Murray, 1972) gave additional support to this hypothesis.

Several other reports of increased mucosal permeability in *N. brasiliensis* infected rats were not in agreement with the timing of the leak lesion as described by Murray *et al.* (1971a). Thus, Neilson (1969) reported that blood loss into the gut lumen became evident on the fourth day after infection and continued until the worms were expelled. Jarrett *et al.* (1967) noted a similar time course for the perivascular leak of intravenously injected carbon.

The experiments described in chapter 5 showed that the rise in mast cells occurred at a late stage of the final expulsive phase in 4 different strains of rats. Because the timing of these responses was different in each strain, it was of interest to re-examine the relationship of the mast cell response to mucosal permeability. The results show that the increase in permeability is related to the worm burden and not to worm expulsion.

RESULTS

Plasma clearance and appearance of Evan's blue in the gut lumen of normal and infected rats

Sixty Wistar rats were infected with 3000 L_3 and were given Evan's blue intravenously 8 or 11 days later. Groups of 5 animals were killed, 5, 10, 30, 60, 120, 240 and 480 min. after injection of the dye and the amount of Evan's blue in the plasma and the gut lumen was measured. An additional group of 30 uninfected rats was examined at similar time intervals after injection of Evan's blue. The results are shown in Figure 7-1.

The amount of Evan's blue in the gut lumen of infected animals was 3-4 times greater than that of normal animals. The dye initially accumulated in the small intestinal lumen and appeared at a later stage in the large intestine both in normal and infected animals. This suggests that the small intestine is the major site of mucosal permeability.

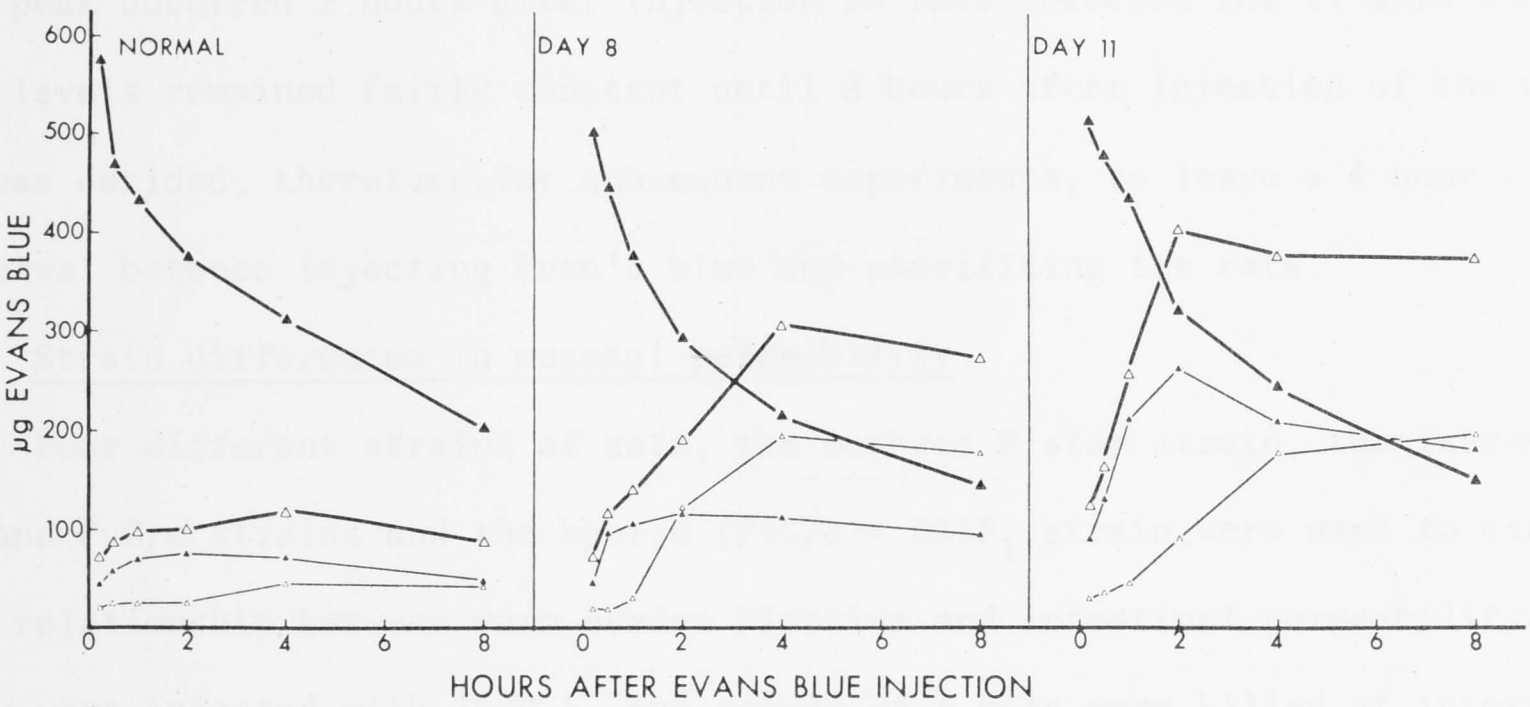


Fig. 7-1: The plasma clearance and appearance of Evan's blue in the gut lumen in normal and infected rats. Wistar rats were infected with 3000 L_3 and mucosal permeability was measured on days 8 and 11. Plasma clearance of Evan's blue (▲ ▬ ▲) is expressed as µg dye/ml of plasma. The quantity of Evan's blue appearing in the small (▲ —— ▲) and large (Δ —— Δ) intestines was measured 15, 30, 60, 120, 240 and 480 min. after i.v. injection of dye. The total amount of Evan's blue in the gut lumen (Δ ▬ Δ) was calculated and is expressed as µg of dye. Each point represents the mean of 5 rats. For clarity, the standard error bars have been omitted.

Evan's blue accumulation in the whole intestine reached a peak 4 hours after injection both in normal rats and in rats infected for 8 days, whereas the peak occurred 2 hours after injection in rats infected for 11 days and the levels remained fairly constant until 8 hours after injection of the dye. It was decided, therefore, for subsequent experiments, to leave a 4 hour interval between injecting Evan's blue and sacrificing the rats.

Strain differences in mucosal permeability

Four different strains of rats, the outbred Wistar strain, the inbred DA and PvG/c strains and the hybrid (PvG/c × DA)F_1 strain, were used to examine the relationship between worm burden kinetics and intestinal permeability. They were infected with 3000 L_3 and groups of 5 rats were killed at intervals after infection for worm counts and for histological studies. An additional 5 infected animals were killed in parallel, 4 hours after injecting Evan's blue, for the measurement of mucosal permeability. At least 5 uninfected animals from each strain were sacrificed in order to measure mucosal permeability in normal rats. Since it was evident from the previous experiment (Fig. 7-1) that Evan's blue was found both in small and large intestines 4 hours after injection, the total amount of Evan's blue in the lumen of the whole gut was used as the index of intestinal permeability. The results are summarized in Figure 7-2.

Marked strain differences were found both in worm burden kinetics and in intestinal permeability. An increase in mucosal permeability occurred early during infection and in all instances the maximum leak preceded the rapid phase of worm expulsion (Fig. 7-2).

These results suggested that the amount of plasma protein in the gut lumen might be related to the worm burden. To investigate this possibility and to determine whether increased mucosal permeability persisted in the absence of the parasites, two further experiments were carried out.

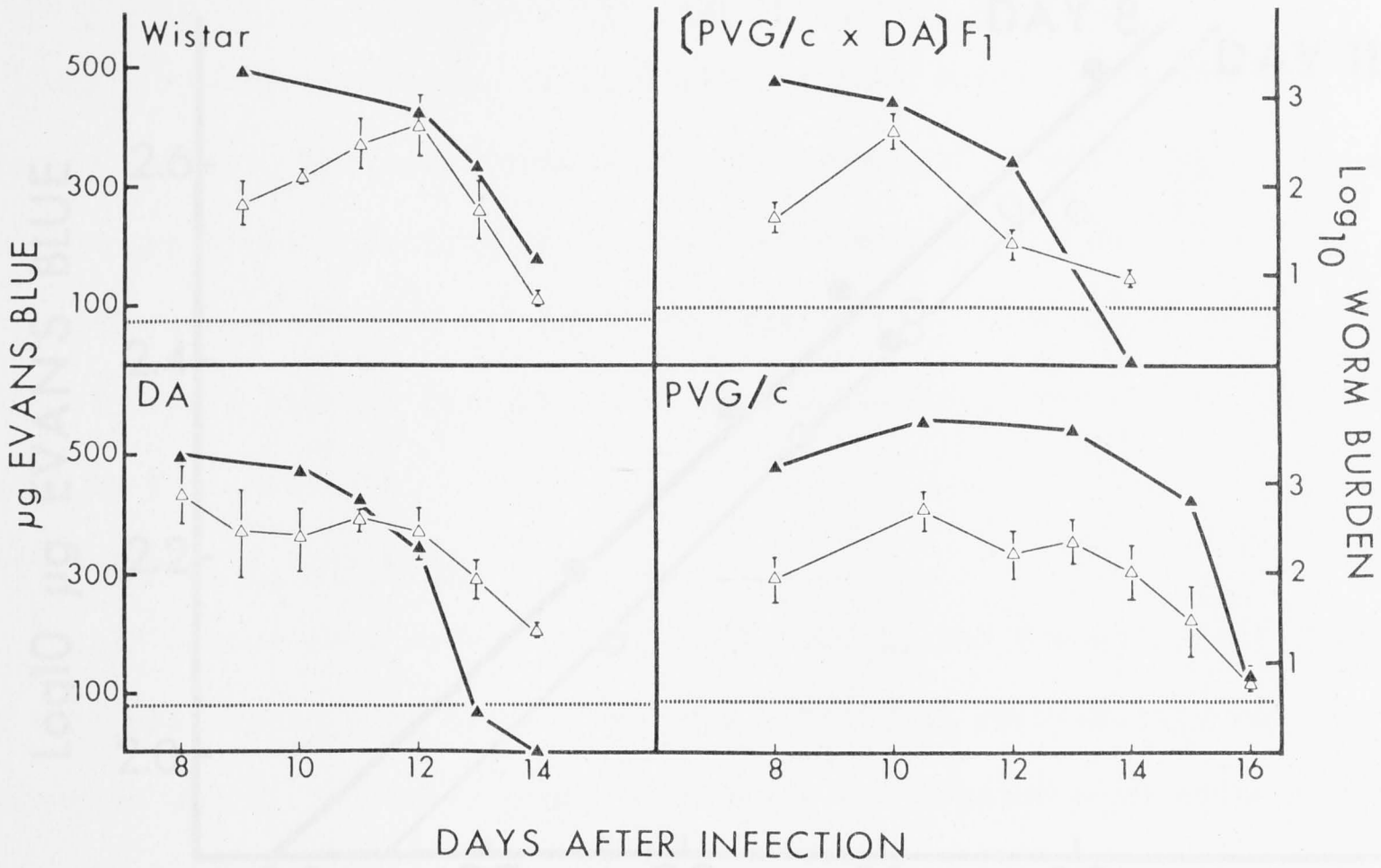


Fig. 7-2: The kinetics of mucosal permeability (Δ —— Δ) and of the worm burdens (▲ —— ▲) in different strains of rats. Mucosal permeability is expressed as the quantity of Evan's blue (µg) in the whole intestine. (The vertical bars express the standard error of the mean for groups of 5 rats). Worm burdens are shown as $\log_{10}$ geometric mean for groups of 5 rats but the standard error bars are omitted for clarity. The amount of Evan's blue in the gut of non-infected rats from each strain was measured in a minimum of 5 rats per strain and the mean and standard error is expressed by (---).

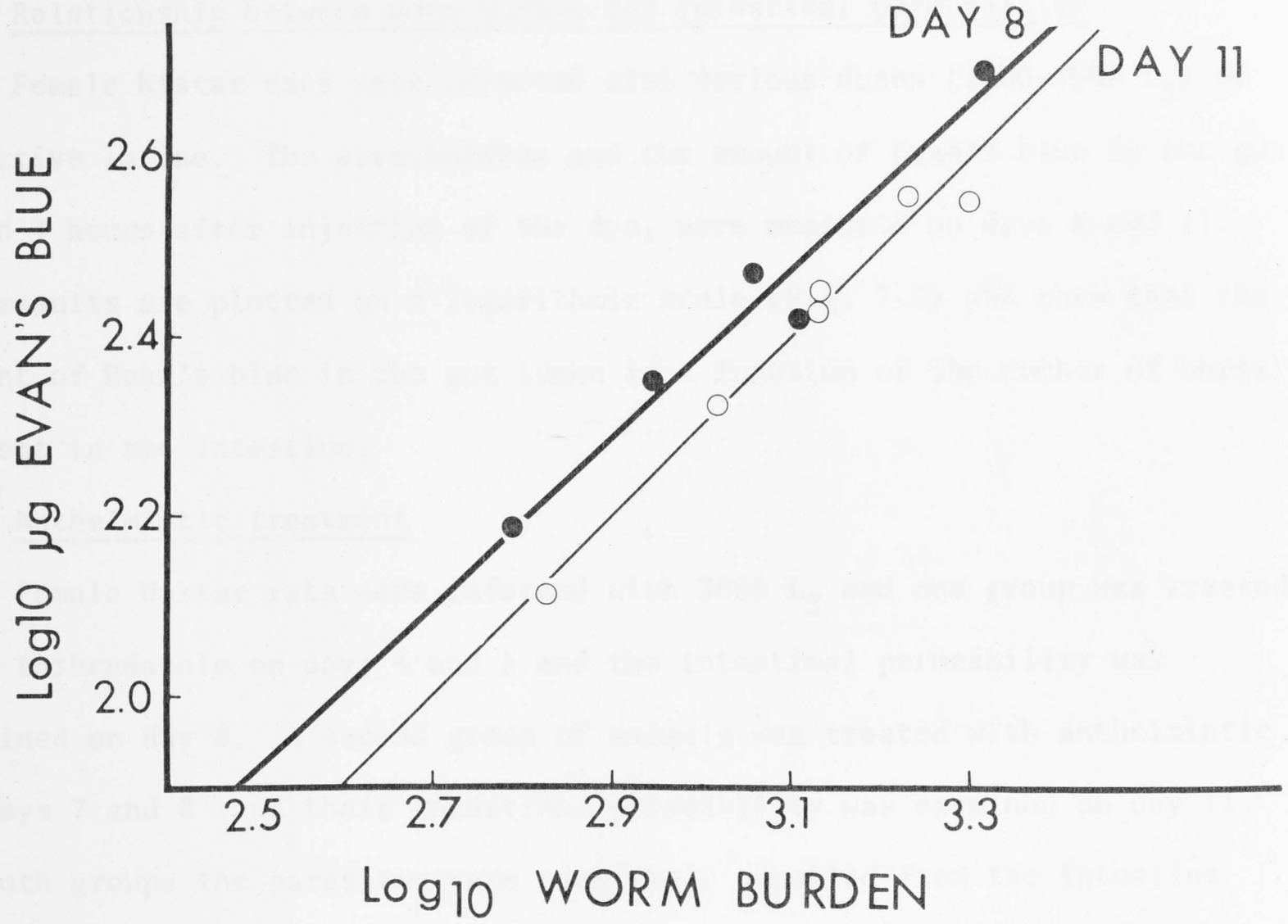


Fig. 7-3: Dose-response relationships between the quantity of Evan's blue in the gut lumen and the worm burdens in Wistar rats 8 and 11 days after infection with different doses of larvae (L_3). Each point represents the mean $\log_{10}$ µg of Evan's blue plotted against the $\log_{10}$ worm burden from groups of 5 rats 8 days after (● —) and 11 days after infection (O —). Regression coefficient for day 8 is 0.9299 ± 0.1236 and for day 11 is 0.9913 ± 0.0831.

Relationship between worm burden and intestinal permeability

Female Wistar rats were infected with various doses (1000-4000 L_3) of infective larvae. The worm burdens and the amount of Evan's blue in the gut lumen 4 hours after injection of the dye, were measured on days 8 and 11. The results are plotted on a logarithmic scale (Fig. 7-3) and show that the amount of Evan's blue in the gut lumen is a function of the number of worms present in the intestine.

Anthelmintic treatment

Female Wistar rats were infected with 3000 L_3 and one group was treated with thibendazole on days 4 and 5 and the intestinal permeability was examined on day 8. A second group of animals was treated with anthelmintic on days 7 and 8 and their intestinal permeability was examined on day 11. In both groups the parasites were completely expelled from the intestine within 24 hours and, as shown in Table 7-1, the amount of Evan's blue in the gut lumen of drug treated animals decreased to the level of normal uninfected controls.

DISCUSSION

The present results establish that there is a marked increase in mucosal permeability during infection with *N. brasiliensis*. There is, in addition, a considerable strain difference both with regard to worm burden kinetics and to intestinal permeability. The dose-response experiments and the results of drug treatment suggest that increased mucosal permeability is a function of the worm burden. However, the increased permeability was related neither to the rapid phase of worm expulsion nor to the rise in mast cell numbers (see chapter 5).

Murray and coworkers (Jarrett *et al.*, 1970; Murray *et al.*, 1971a; Rev. by Murray, 1972) described a striking correlation between the intestinal mast

Table 7-1: Effect of anthelminthic treatment on the mucosal permeability of rats infected with *N. brasiliensis*.

	TBZ Treatment	µg Evan's blue
Normal	-	116.2 ± 2.9
Day 8	-	201.7 ± 16.7
	+	92.6 ± 6.4
Day 11	-	276.3 ± 25.3
	+	104.1 ± 5.1

Wistar rats were infected with 3000 L_3 and thiabendazole (TBZ) was given by stomach tube to one group on days 4 and 5 and the rats were killed on day 8. The second group was treated on day 7 and 8 and the rats were killed on day 11. Control rats were given saline by stomach tube. Each animal was injected with 0.5 ml of 1% Evan's blue and the amount of the dye in the whole length of the intestine was measured 4 hours later. Each value represents the mean ± standard error of 5 rats.

cell response, the increase in mucosal permeability and the rapid phase of worm expulsion. These findings, together with ultrastructural observations (Murray *et al.*, 1969; Murray, 1970), led them to propose that a mast cell mediated 'leak lesion' allowed anti-worm antibody across to the parasites, thus causing worm expulsion.

On the other hand, the present experiments, in agreement with several other reports (Jarrett *et al.*, 1967; Neilson, 1969), indicated that increased mucosal permeability was not related to the rapid phase of worm expulsion. Furthermore, the intestinal mast cell response was found to occur only at a stage where the worm burden was markedly reduced (see chapter 5). These findings strongly suggest that the mast cell mediated 'leak lesion' hypothesis is no longer tenable.

There may be several reasons for the difference between the present results and those of Murray *et al.* (1971a). The latter group reported a mucosal leak of 1 ml of plasma every 4 hours in normal rats increasing to 5 ml every 4 hours during worm expulsion. These unphysiological values were calculated without reference to a plasma clearance curve for PVP nor was the rate of appearance of PVP in the gut lumen determined. Moreover, different markers were used in each experiment. Evan's blue has been shown to combine preferentially with plasma albumin (Rawson, 1943) so that the measurement of this dye in the gut lumen to a large extent reflects the translocation of albumin molecules whereas ^{131}I-PVP is a heterogenous polymer with an average molecular weight of 40,000. The distribution and rate of catabolism of this polymer are different from those of albumin, hence the excretion of ^{131}I-PVP into the intestinal lumen does not quantitatively measure the gastrointestinal leakage of plasma protein (Jeejeebhoy and Coghill, 1961; Rev. by Jeffries *et al.*, 1962).

Plasma protein leak into the gastrointestinal tract is a complex phenomenon with various factors involved. Vascular permeability, intestinal epithelial permeability, the haemodynamics in the mucosa, intestinal motility and the amount and consistency of ingested material may affect not only the translocation of plasma proteins across the intestinal mucosa but also the rate of their passage into the large intestine. In the present experiments, the amount of Evan's blue in the whole gut was measured and this reflects the combined vascular and epithelial leak. Unfortunately, the contributions of each component to the whole cannot be distinguished by this technique.

The extent of the increased permeability was proportional to the number of worms present in the intestine. Moreover, anthelmintic treatment brought about a decrease of the plasma leakage even though this drug does not prevent a rise in mast cell numbers (Murray, *et al.*, 1971a). These findings strongly suggest that the increased permeability of the small intestine during *N. brasiliensis* infection is a function of the mechanical or toxic damage to the epithelial cells and/or the vascular bed caused by the parasites themselves and/or their metabolites.

Local anaphylaxis brought about by immunoglobulins and by cells other than IgE and mast cells cannot at present be ruled out as a cause of increased mucosal permeability. The roles of basophils, eosinophils and enterochromaffin cells, which contain biologically active mediators, are not known nor is it clear what function rat IgG_2a has in the response. This class of immunoglobulin has homocytotropic properties (Stechschulte, Orange and Austen, 1971) and Ogilvie and Jones (1969) suggested that 4 hour PCA antibodies, probably IgG_2a, occurred in rats immunized with *N. brasiliensis* worm extracts. Moreover, Jarrett and Stewart (1973) found that active cutaneous anaphylaxis preceded the appearance of circulating IgE antibody in *N. brasiliensis* -infected rats. Since West (1959) has reported that

neither histamine nor mast cells participated in the anaphylactic response in rats, the possibility that increased mucosal permeability is mediated by hitherto unrecognized anaphylactic mechanisms cannot be excluded.

CHAPTER 8

THE INTESTINAL IgA RESPONSE

CHAPTER 8

THE INTESTINAL IgA RESPONSE

Secretory IgA has an important role in the protection of mucosal surfaces (Rev. by Heremans, 1968, 1974; Tomasi and Gray, 1972) and large numbers of plasma cells which synthesize IgA are found in the intestinal lamina propria (see chapter 1). These cells are, to some extent, derived from lymphoblasts in the TDL, some of which originate from Peyer's patches (Rev. by Lamm, 1976 and by Waksman and Ozer, 1976).

Although *N. brasiliensis* is expelled from the intestine as a result of an immune response by the host, the contribution of specific antibodies of the IgA class to this phenomenon has only been partially characterised. Jones *et al.* (1970) demonstrated that the passive transfer of an IgA fraction from immune sera occasionally had some protective activity against implanted adult worms. Haemagglutinating antibody against extracts of adult worms was demonstrated in the intestinal fluid of infected rats and identified as IgA by gel filtration (Poulain *et al.*, 1976a). Also some protective activity was demonstrated after oral immunisation with worm metabolites or worm extracts (Poulain *et al.*, 1976b). These findings suggest that IgA antibody could be involved in worm expulsion.

Protection against adult *N. brasiliensis* can be adoptively transferred by immune TDL (chapter 3 and Ogilvie *et al.*, 1977) and, after fractionation of the TDL, effector cells were present only in the sIg^- subpopulation from day 10 TDL and predominantly in the sIg^- subpopulation from hyperimmune TDL (chapter 4). These results raise the possibility that IgA antibody synthesis may be regulated by adoptively transferred 'helper' T-cells. A small but significant degree of protection was, however, provided by the sIg^+

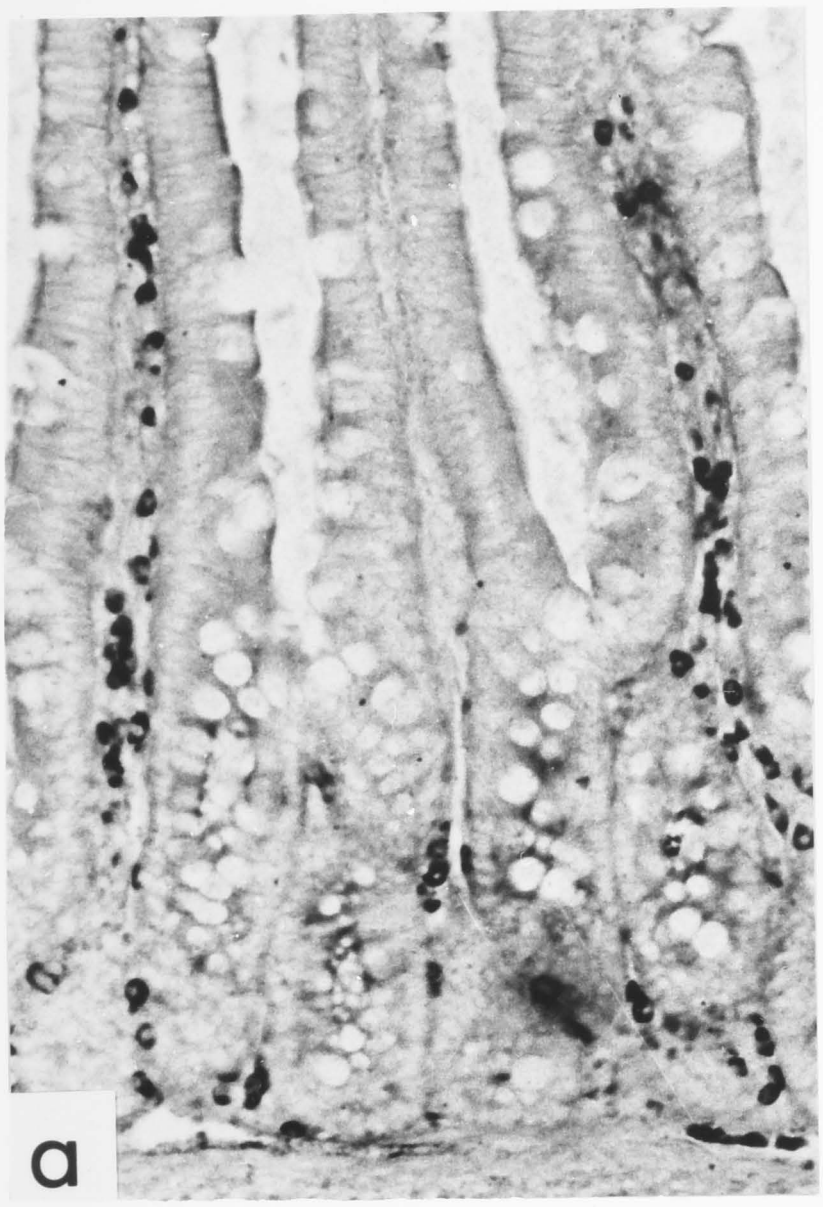
Fig. 8-1: Light micrographs of the intestinal mucosa of (PvG/c × DA)F_1 rats. IgA-synthesizing cells are demonstrated by the immunoperoxidase technique. Sections were incubated with rabbit anti-rat IgA antiserum then with sheep Ig anti-rabbit $F(ab')_2$-HPO.

(a) The intestinal mucosa of a normal rat (× 200).

(b) The intestinal mucosa of a rat 8 days after infection with 4000 L_3. IgA-synthesizing cells have almost completely disappeared. (× 200).

(c) The intestinal mucosa of a rat 10 days after infection with 1000 L_3 and adoptive immunization with 1 × 10^8 hyperimmune TDL. (× 200).

(d) High power magnification of a different field from the same section as (c). Many IgA-synthesising cells are seen in the lamina propria. (× 500).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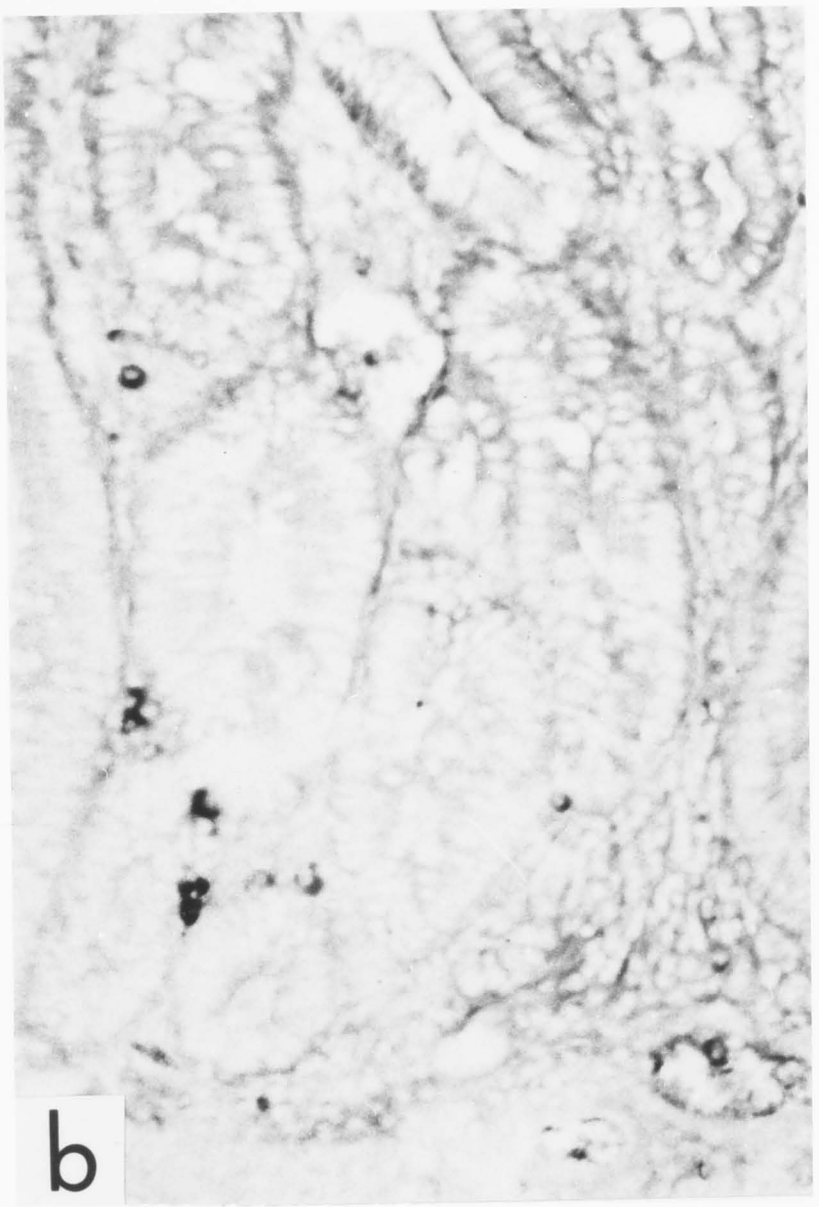
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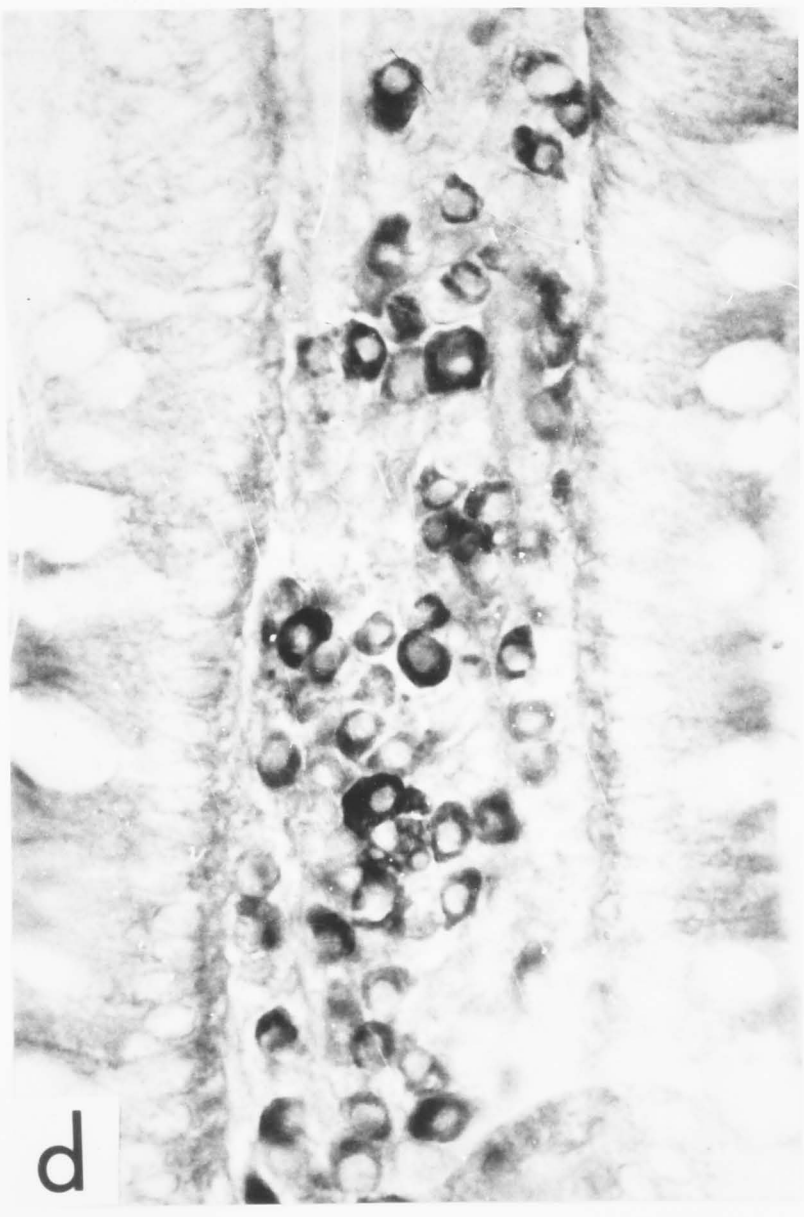

d

subpopulation from hyperimmune TDL (chapter 4) and there was, in addition, a small proportion of Ig-synthesizing cells, possibly of the IgA class, in the sIg^- cell populations.

The present study was undertaken to examine the effects of adoptively transferring immune TDL on the IgA-synthesizing cells in the intestinal lamina propria in rats infected with *N. brasiliensis*. The results show that such cells increase in number after adoptive transfer of hyperimmune but not day 10 TDL.

RESULTS

Kinetics of IgA-synthesizing cells during primary infection

$(PvG/c \times DA)F_1$ rats were infected with 1000 or 4000 L_3 and IgA synthesising cells in the lamina propria were examined at various times after infection. Very few remain in the lamina propria (Fig. 8-1b) on days 6 and 8 of infection with 4000 L_3 (Fig. 8-2) but they increase in number by day 10 and return almost to normal levels (22.6 ± 3.4/VCU, also see Fig. 8-1a) on days 12 and 14. No significant change in the number of IgA-synthesizing cells was observed in rats infected with 1000 L_3 (Fig. 8-2).

The virtual absence of IgA-synthesizing cells on day 8 of a 4000 L_3 infection was indirectly confirmed by a study of the immunoelectrophoretic pattern of the intestinal contents. Intestinal washings were collected from rats 8, 10, 12 and 14 days after infection by flushing the small intestine with 5 ml of PBS. The contents were homogenized, centrifuged at 20,000 g and concentrated 10 times in a Minicon (Amicon) ultrafiltration unit. Two microlitres samples of concentrated intestinal fluid were electrophoresed and precipitin lines were allowed to develop with a rabbit-anti-whole rat serum and a goat anti-rat IgA serum provided by Dr. H. Bazin. Whereas IgA was present in the intestinal fluid of normal rats, it was not detected in

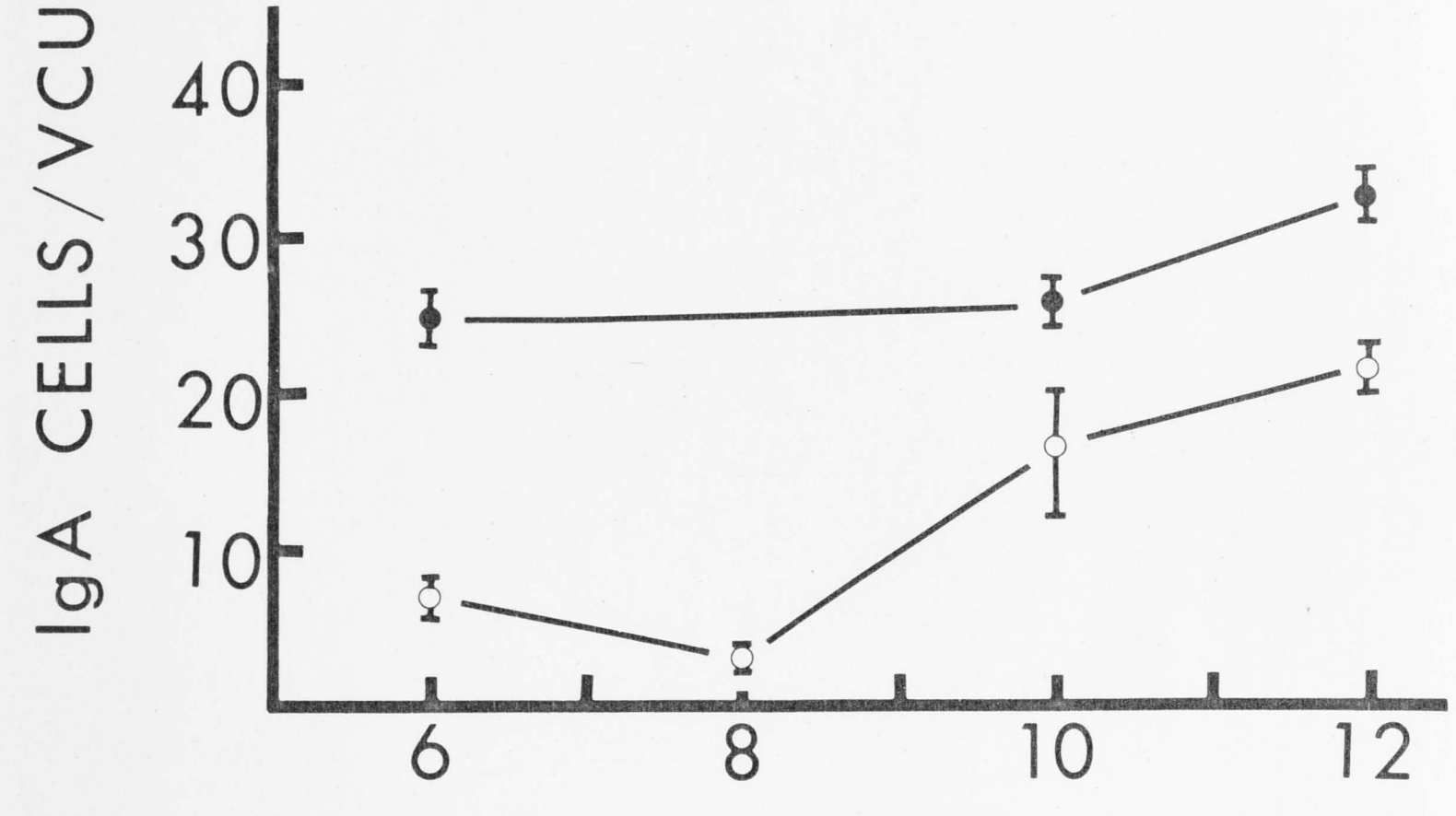
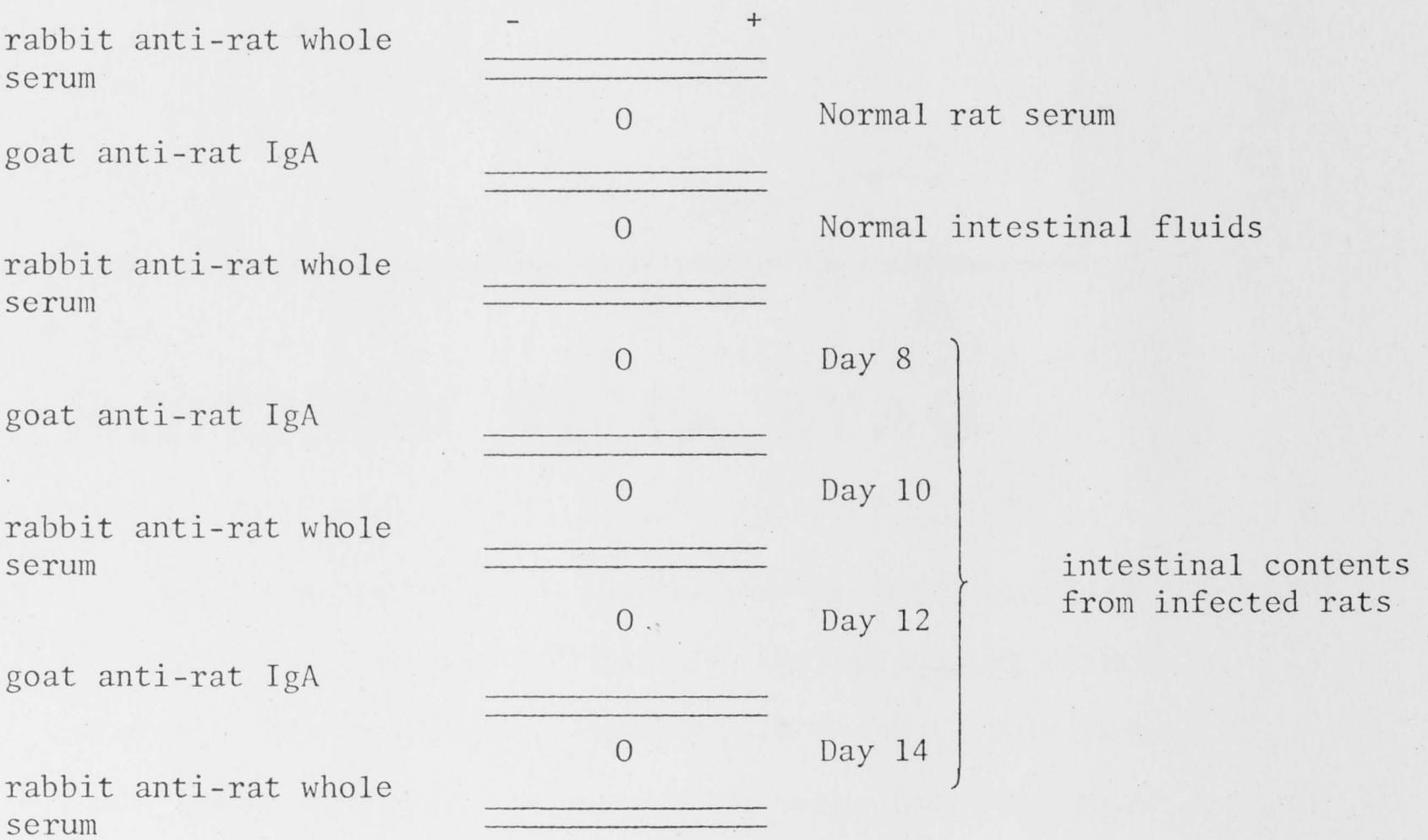
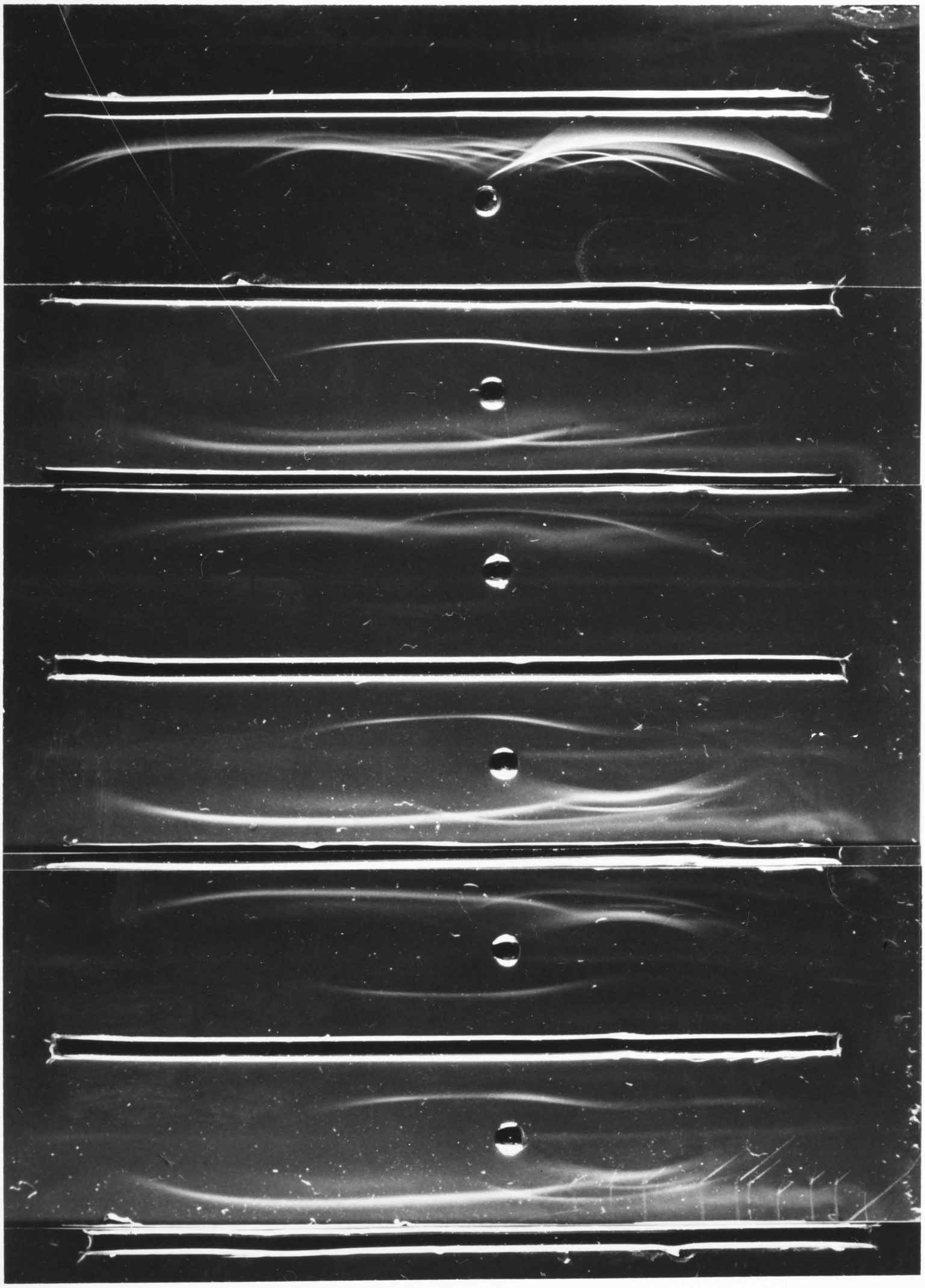


Fig. 8-2: The kinetics of IgA-synthesising cells in the intestinal lamina propria of rats infected with 1000 L_3 (● —— ●) or with 4000 L_3 (o —— o). Each point represents the arithmetic mean ± standard error for a group of 5 rats. In normal rats there were 22.6 ± 3.4 IgA cells/VCU.

Fig. 8-3: Immunoelectrophoretic analysis with goat anti-rat IgA and with rabbit anti-rat whole serum of the intestinal contents from normal rats or from infected rats 8, 10, 12 and 14 days after infection with 4000 L_3. Normal rat serum was electrophoresed as a control.





the intestinal fluid obtained 8 days after infection with 4000 L_3 but it re-appeared on day 10 and was present on days 12 and 14 (Fig. 8-3). In addition, IgG and alpha-globulin were also detected in the intestinal fluids both of normal and of infected animals (Fig. 8-3).

Effect of adoptive immunisation

Since the adoptive transfer of immune TDL causes an earlier expulsion of adult worms from the intestine (chapter 3), IgA-synthesizing cells in the lamina propria in infected recipients of immune TDL were examined. The experimental protocol was described in detail elsewhere (chapter 3). Briefly, (PvG/c × DA)F_1 rats were infected with 1000 L_3 and, at the same time, were given 1×10^8 day 10 TDL or hyperimmune TDL obtained 1 week after tertiary infection. The results of these experiments are shown in Fig. 8-4. In the control groups which were not given cells, the number of IgA-synthesising cells remained contant (Fig. 8-4 Top) or increased slightly at the time of worm expulsion (Fig. 8-4 Bottom). Adoptive transfer of day 10 TDL had no effect on the number of IgA-synthesizing cells in the lamina propria (Fig. 8-4 Top) whereas hyperimmune TDL caused a significant ($P < 0.01$) increase in their number on days 8 and 10 (Fig. 8-4, Bottom, also see Fig. 8-1c, d).

Dose-response relationship

To determine whether there was any relationship between IgA-synthesizing cells in the lamina propria and the transferred immune TDL, a range of between 1×10^7 and 4×10^8 day 10 TDL were injected into recipients at the time of a 1000 L_3 infection. For the purpose of counting the worm burdens (chapter 3) and to obtain histological specimens for the detection of IgA, all animals were killed 8 days after infection and transfer of cells. The results show that even at the highest dose (4×10^8),

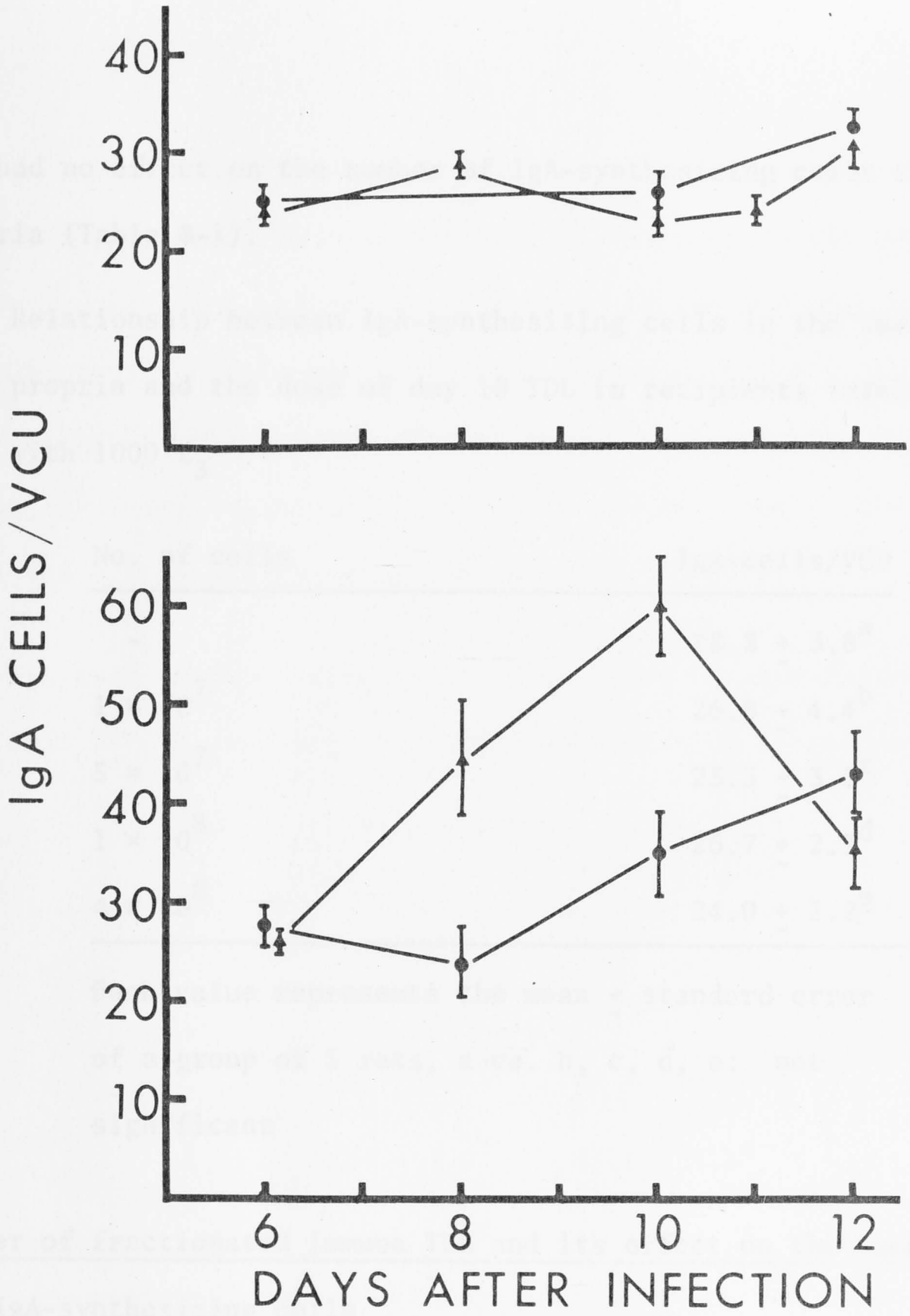


Fig. 8-4: Kinetics of IgA-synthesising cells in the intestinal lamina propria of rats adoptively immunised with immune TDL and infected with 1000 L_3. Top: adoptive immunisation with day 10 TDL (▲ — ▲); bottom: adoptive immunisation with hyperimmune TDL (▲ — ▲). Infected controls were not given cells (● —— ●). Each point represents the arithmetic mean ± standard error for a group of 5 rats. In normal rats there were 22.6 ± 3.4 IgA-cells/VCU.

day 10 TDL had no effect on the number of IgA-synthesizing cells in the lamina propria (Table 8-1).

Table 8-1: Relationship between IgA-synthesizing cells in the lamina propria and the dose of day 10 TDL in recipients infected with 1000 L_3

No. of cells	IgA-cells/VCU
-	18.8 ± 3.8^a
1×10^7	26.8 ± 4.4^b
5×10^7	23.3 ± 3.0^c
1×10^8	26.7 ± 2.7^d
4×10^8	24.0 ± 2.2^e

Each value represents the mean $\pm$ standard error of a group of 5 rats, a *vs.* b, c, d, e: not significant

Transfer of fractionated immune TDL and its effect on the number of intestinal IgA-synthesizing cells

Immune TDL obtained from donors 10 days after primary infection or 1 week after tertiary infection were fractionated into sIg^- and sIg^+ cell populations (chapter 4). The original cell population, the sIg^- and the sIg^+ cells, or a population reconstituted with sIg^- and sIg^+ cells were injected intravenously into 4 groups of recipients which were infected with 1000 L_3 at the same time. Infected controls were given Hank's-BSA. The experimental protocol and the worm burden kinetics in this experiment were described in detail in chapter 4 (see Tables 4-1, 4-2 and 4-3). The results of the IgA-response are summarized in Table 8-2. None of the cell populations had any

effect on the number of IgA-synthesizing cells in the lamina propria. On the other hand, hyperimmune TDL caused a slight increase in the number of IgA-synthesising cells and, after fractionation, it was the sIg^+ population which was found to adoptively confer the IgA response (Table 8-2).

When hyperimmune TDL were transferred into normal animals, the number of intestinal IgA-synthesizing cells (21.5 ± 2.1) was the same as that of the normal (22.6 ± 3.4/VCU) and infected controls (25.3 ± 1.1).

Table 8-2: IgA-synthesizing cells in the intestines of rats infected with 1000 L_3 and injected with subpopulation of immune TDL

	IgA-synthesizing cells/VCU	
	Day 10 TDL	HITDL (1w)
-	24.5 ± 1.2[a]	25.3 ± 1.1[a]
sIg^-	24.2 ± 1.7[b]	26.7 ± 1.4[b]
sIg^+	23.2 ± 1.6[c]	34.6 ± 3.2[c]
sIg^- / sIg^+	23.7 ± 3.4[d]	35.6 ± 3.8[d]
Original	23.7 ± 1.8[e]	36.1 ± 3.2[e]
	a *vs.* b, c,d,e not significant	a *vs.* b not significant a *vs.* c,d,e $0.05 > P > 0.02$

Each value represents the mean ± standard error of a group of 5-6 rats. Details of the number of cells transferred and the worm burdens in each group have been given in Tables 4-2 and 4-3.

DISCUSSION

IgA-synthesizing cells in the lamina propria of rats infected with *N. brasiliensis* were increased in number after the adoptive transfer of

immune TDL obtained from donors 1 week after a tertiary infection. Fractionation of these hyperimmune TDL by the rosetting procedure of Parish and Hayward (1974a) revealed that the sIg^+ subpopulation was responsible for this increase. On the other hand, day 10 TDL, which were more efficient than hyperimmune TDL in causing worm expulsion after adoptive transfer, had no detectable effect on the number of intestinal IgA-synthesizing cells even when large numbers of cells (4×10^8) were transferred. Infection with 4000 L_3 caused a severe depletion of IgA-synthesizing cells in the lamina propria but the numbers rose to normal levels just prior to worm expulsion. On the other hand, infection with 1000 L_3 had little or no effect on the number of IgA-synthesising cells found in the intestine.

Since Gowans and Knight (1964) reported that the large lymphocytes in the thoracic duct lymph primarily localized in the intestinal lamina propria, it has been established beyond doubt that at least some IgA-producing plasma cells are derived from blast cells in the thoracic duct lymph which contain IgA and/or have IgA on their surface (Williams and Gowans, 1975; Rev. by Lamm, 1976, Rev. by Waksman and Ozer, 1976). The present finding that intestinal IgA-synthesizing cells increase in number following the adoptive transfer of sIg^+ cells from hyperimmune TDL would support the concept that TDL contain IgA plasma cell precursors.

Although the localization of blast cells from TDL in the gut mucosa is probably antigen-independent (Griscelli *et al.*, 1969; Halstead and Hall, 1972; Guy-Grand *et al.*, 1974; Parrott and Ferguson, 1974), it has been suggested that the homing patterns of such cells along the intestinal tract is affected by local inflammatory responses (Parrott, Rose, Sless, Freitas and Bruce, 1976; Pierce and Gowans, 1975). The present results show that the adoptive transfer of hyperimmune TDL causes an increase of intestinal IgA-synthesizing cells in infected recipients but not in normal control animals.

This would suggest either that there is non-specific accumulation of IgA-synthesizing cells in the inflamed areas of the gut or that the increased population may be specific antibody-forming cells which, after adoptive transfer, underwent proliferation and maturation in the lamina propria in response to further antigenic stimulation.

The number of IgA-synthesizing cells in the intestine decreased 6 and 8 days after infection with 4000 L_3 suggesting that *N. brasiliensis* adult worms release some cytotoxic substances which destroy such cells. Hunter and Leigh (1961) demonstrated that anti-sheep red cell agglutinating antibody production was suppressed by *N. brasiliensis* infection and it is possible that some component(s) of the worm metabolites may have non-specific deleterious effects on antibody-forming cells.

Finally, any protective role for IgA antibody against the parasites remains uncertain. The effector cells which cause worm expulsion were found only in the sIg^- cell population of day 10 TDL and predominantly in the sIg^- cell population of hyperimmune TDL (chapter 4) and these cells had no detectable effect on the intestinal IgA response. This observation reduces but, for a variety of reasons, does not rule out a role for IgA in worm expulsion. It is possible, for example, that transferred 'helper' T cells enable precursor cells to become active specific IgA antibody-forming cells and that these form a relatively small proportion of the total population. Alternatively, the turn-over of IgA plasma cells may increase such that a relatively high proportion of antibody-forming cells are present even though the total number of cells remains constant. The small proportion of IgA-synthesizing cells found in the sIg^- cell population (chapter 4) may also provide specific antibody-forming cell precursors which could proliferate at the site of infection. It is clear, however, that the sIg^+ cell population from hyperimmune TDL, which increased the number of intestinal IgA-

synthesizing cells present in the lamina propria, did have some protective effect (chapter 4). This finding suggests that specific antibody of the IgA class might contribute to protection although it is now known whether this would be true for primary or for secondary infections. Further recognition of any definitive role for IgA in worm expulsion must await techniques which permit the visualisation of specific anti-worm antibody-forming cells.

CHAPTER 9

FINAL DISCUSSION AND CONCLUSIONS

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The effector mechanisms which bring about the expulsion of the intestinal parasite *N. brasiliensis* were investigated with the aim of studying several aspects of mucosal immunity. The adoptive transfer of TDL from infected donors was used to manipulate the various mucosal immune responses and its effect on the worm burden kinetics and on cellular changes in the mucosa were examined and compared.

The results of these experiments establish that TDL drained from donor rats during the rapid phase of worm expulsion (i.e. days 10-15 after primary infection) are more effective than any other source of immune cells. Dose-response experiments show that there is a direct and inverse relationship between the number of cells transferred and the number of parasites remaining in the intestine and that day 10 TDL are significantly more effective than hyperimmune TDL in conferring immunity. These day 10 TDL confer a high degree of protection not only against adult worms established by larval infection but also against transplanted 'normal' and 'damaged' worms.

Immune TDL were separated into cells lacking sIg or cells bearing sIg and the protective capacities of these two subpopulations were tested. The effector cells which caused worm expulsion were found only in the sIg^- cell population from day 10 TDL. When hyperimmune TDL were fractionated and transferred, the effector cells were also predominantly found in sIg^- cell populations, although a small but significant degree of protection was also conferred by the sIg^+ cell population. Reconstitution of these subpopulations showed that with hyperimmune TDL the protective capacity of the combined population was greater than that of either subpopulation alone.

These results confirm and extend the observation by Ogilvie *et al.* (1977) who demonstrated that the expulsion of transplanted 'damaged' worms from the intestines of heavily irradiated rats could be induced by immune TDL or MLNC. The effector cells appeared as early as 8 days after primary infection and, after fractionation of the TDL, were found in the sIg^- cell population. The present results clearly show that not only 'damaged' worms but also 'normal' worms and worms established by larval infection are susceptible to the action of adoptively transferred immune TDL. They do not therefore, agree with the currently held view that worms must first be damaged by antibody before they can be expelled by the action of immune cells (Ogilvie and Love, 1974).

The data concerning the adoptive response against *N. brasiliensis* are conflicting, mainly because the dose and source of cells, the immune status of the cell donor, the strain of rats used and the dose of infective larvae or of adult worms have not been standardised. The present experiments establish some of these conditions and emphasise the importance of kinetic and dose-response studies in evaluating the efficacy of transferred cells. Furthermore, in the present experiments, TDL obtained from hyperimmune donors were consistently less effective than those obtained from donors 10 days after primary infection. In contrast, studies on the worm burden kinetics in actively immunized rats (Jarrett and Urquhart, 1971) show that immunity against the parasites increases with repeated infections. This discrepancy between adoptive immunisation and actively acquired immunity suggests that quantitatively and/or qualitatively, different protective mechanisms are involved in primary and secondary responses against the parasite. This is also supported by the present results where sIg^+ sub-populations fractionated from hyperimmune donors conferred some protection whereas sIg^+ cells fractionated from donors cannulated on day 10 of a primary infection did not.

The hypothesis that a 'leaky mucosa' induced by local anaphylaxis and mediated by mast cells and IgE antibody may cause worm expulsion (Murray, 1972) was also investigated. Kinetic studies showed that the rise in IMC numbers was closely related only to the final stage of the rapid phase of worm expulsion, whereas the circulating IgE antibody response against this parasite was detected after worm expulsion. This dissociation of the IMC and IgE responses does not support the proposition that local analphylaxis is important for protection, but perhaps the strongest argument against the 'leak lesion' hypothesis was the evidence that increased plasma protein leakage occurred well before the worms were expelled and appeared to be a function of the worm burden. The failure to relate the IgE antibody response to the IMC response, to intestinal permeability and to worm expulsion appears to rule out a role for local anaphylaxis in worm expulsion during a primary infection. However, because increased numbers of IMC are present even 1 month after a primary infection (Miller, 1969) and there is an elevation of the IMC response (Whur, 1966) and of IgE titres (Jarrett *et al.*, 1976) during a secondary infection, IgE and mast cells may be important in protection against secondary infections.

Finally, the possibility that local synthesis of IgA contributes to worm expulsion has been examined using an immunocytochemical technique. The results show that infection with 1000 L_3 has little or no effect on the number of IgA-synthesising cells in the intestinal lamina propria, whereas infection with 4000 L_3 causes severe depletion of such cells. However, in the latter situation the number of IgA cells returns to normal prior to worm expulsion. Adoptive transfer of hyperimmune TDL causes an increase in the number of IgA cells in the lamina propria and this response is transferred by the sIg^+ cells from hyperimmune TDL. On the other hand, adoptive transfer

of day 10 TDL had no effect on the number of IgA-synthesising cells in the mucosa. Because specific anti-worm antibody-forming cells were not examined, these results do not exclude a role for IgA in primary infections although they do suggest that IgA antibody may contribute to protection against a secondary infection. Poulain *et al.* (1976b) demonstrated haemagglutinating antibodies of the IgA class within the intestinal lumen early in a primary infection but whether this antibody has any protective activity is not known.

The adoptive transfer of immune TDL raises some interesting questions concerning the origin of intestinal mast cells and of IgA- and IgE-synthesising cells and the mechanisms which regulate their development.

The intestinal mast cell response is most effectively transferred by sIg^- cells from day 10 TDL but only when the recipients are harbouring parasites. These results, together with recent findings that infected athymic (*nu/nu*) mice are able to mount an IMC response during infection with *N. brasiliensis* only when restored by thymic implantation (Olson and Levy, 1976), strongly suggest that intestinal mast cells are under control of the thymus. The present results suggest that IMC could originate from adoptively transferred sIg^- cells from immune TDL. The *in vitro* experiments by Ginsburg and Lagunoff (1967) in which mast cells were observed to develop in cultures of TDL from mice hyperimmunized with protein antigen support this concept. Alternatively, immune TDL may provide 'helper' cells for the *in situ* differentiation of IMC from undifferentiated lymphoid blast cells (Miller, 1969; 1971a). Since several *in vitro* experiments (Ginsburg and Sachs, 1963; Ishizaka *et al.*, 1976) suggest that mast cells can arise from normal thymus cells cultured on fibroblast monolayers without any specific antigen stimulation, the requirement for antigen and the

importance of specific stimulation in the development of mast cells should be further investigated both *in vivo* and *in vitro*.

Although a circulating IgE antibody response was adoptively transferred by hyperimmune TDL, both sIg^- and sIg^+ cells were required to do this, which would suggest that the IgE antibody-forming cells were of donor origin. Since Ishizaka (1976) has proposed that the generation of IgE-bearing cells may not require T-cells whereas the differentiation of IgE-bearing cells into IgE-forming cells is highly dependent on T-cells and also on antigen, it is reasonable to assume that TDL obtained from hyperimmune donors contain precommitted IgE-bearing cells which can differentiate after antigenic stimulation into IgE-forming cells only with T-cell help. IgE-forming cells are predominantly found in tissues adjacent to mucous surfaces in primates (Ishizaka *et al.*, 1969) but Mayrhofer *et al.* (1976) demonstrated that most of the IgE-containing cells in the intestinal lamina propria of *N. brasiliensis*-infected rats were mast cells and not plasma cells. The adoptive transfer of hyperimmune TDL may, therefore, provide a useful experimental system for the investigation not only of cell traffic amongst IgE precursor cells, but also the mechanisms of IgE antibody production and the biological role of IgE antibodies.

The results in chapter 8 suggest that at least some IgA-synthesizing cells in the intestinal lamina propria are derived from sIg^+ cells in hyperimmune TDL. Since Gowans and Knight (1964) demonstrated that large dividing lymphocytes in rat thoracic duct lymph preferentially localize in the gut-associated lymphoid tissue, there has been considerable accumulation of data concerning the origin and traffic of IgA-synthesizing cells in the lamina propria (Rev. by Lamm, 1976; Waksman and Ozer, 1976). IgA-containing cells in thoracic duct lymph from normal rats also bear surface

IgA (Williams and Gowans, 1975) and Pierce and Gowans (1976) found that IgA-synthesising specific antibody-forming cells appeared in the thoracic duct lymph after oral boosting and that such cells tended to localize in the gut. These findings, together with the present results, strongly suggest that some IgA-synthesising cells and/or their precursors appearing in the thoracic duct lymph of hyperimmune rats have immunoglobulin on their surface and that they preferentially localize in the gut. However, the density of this sIg may not be sufficient to fractionate and separate all IgA-synthesizing cells from sIg^- cells as was evident from the experiments in chapter 4.

Generally speaking, as might be expected, the mucosal defence mechanisms against *N. brasiliensis* in the rat are a complex of host responses including specific immune responses and non-specific inflammation. In the present investigations the effect of adoptive immunization has proved to be a fertile area for investigating the relative importance of various components involved in the protective mechanisms against the parasites. The results following the adoptive transfer of subpopulations of immune TDL have raised a number of points which require further investigation. For example, effector cells which confer protection are found only in sIg^- cells from day 10 TDL whereas they are found predominantly in sIg^- cells and, to a lesser extent, in sIg^+ cells from hyperimmune TDL. IgE and IgA responses can be transferred only by hyperimmune TDL, the former requiring both sIg^+ and sIg^- cells whereas the IgA response was transferred by sIg^+ cells alone. On the other hand, IMC responses were much more effectively transferred by day 10 TDL than by hyperimmune TDL with the effector cells for this response residing in the sIg^- cell population. These observations raise the question as to whether the mechanisms of worm expulsion in primary and secondary infections are essentially the same and, if not, are the differences qualitative or quantitative?

There is a possibility that IgA and IgE antibodies contribute to worm expulsion during secondary infections because passive transfer of hyperimmune sera confers some protection (see chapter 1); systematic studies are, however, necessary to determine the role of antibodies in worm expulsion. Also, the discrepancy between day 10 TDL and hyperimmune TDL with regard to their ability to confer protection, IMC responses and IgA or IgE responses, as well as the relatively poor protection achieved by hyperimmune TDL when compared with active immunization should be further investigated in terms of the traffic of lymphocytes carrying immunological memory. Gowans and McGregor (1965) suggested that memory for antibody production is carried both by circulating and by non-circulating lymphocytes. Similarly, memory helper T-cells appear shortly after antigen stimulation and contribute to a primary response by activating virgin B-cells, whereas development of memory antibody-forming cells precursors takes much longer and requires a larger dose of antigen (Cunningham and Sercarz, 1971). It has also been suggested that memory for homograft rejection is carried by non-recirculating T-lymphocytes (Hall, Dorsh and Roser, 1976). Therefore, the different protective capacities of day 10 and hyperimmune TDL when compared with active immunization may, in part, be due to differences in the generation and traffic of memory cells for each response.

The results in chapter 4 establish that the day 10 effector cells which bring about worm expulsion reside exclusively in the sIg^- subpopulations and that such cells also predominate in the sIg^- subpopulation of hyperimmune TDL, the implication being that T-cells are important in protection against the parasite. The failure to passively protect mice against *N. brasiliensis* with immune serum (Ogilvie, 1971), together with the inability of athymic (*nu/nu*) mice to expel their worms (Jacobson and Reed, 1974) emphasizes the

importance of cellular immune responses against *N. brasiliensis* in this species. The mechanisms of worm expulsion in the rat appear to be more complex as is clear from the present results, yet there is little doubt that thymus-derived lymphocytes are important for protection against this parasite.

The significance of local cell-mediated immunity in the respiratory tract has been reviewed by Waldman and Ganguly (1975) who suggested that T-cell derived 'lymphokines' may be important in dealing with pathogenic microbes. Further observations would be required to apply this concept to the gastrointestinal mucosa. However, since it has been reported that T-cells might induce epithelial cell damage and thus alter villus morphology (Ferguson and Jarrett, 1975) the role of the mucosal epithelium in worm expulsion should be examined in this regard. Recently, we have found that there is, prior to worm expulsion, extensive epithelial cell differentiation to form goblet cells and that this process appears to be regulated by immune lymphocytes (Miller, Nawa and Hughes, 1977). Furthermore, several other cell types such as basophils and eosinophils which are under immune regulation should be examined because of their associations with parasitic infections. This may also be true for epithelial cells such as Paneth cells and enteroendocrine cells.

If a complete understanding of these cellular responses, including antibody production and mediator release is eventually achieved, there may be considerable progress in applied studies of the pathogenesis of gastrointestinal disease and of allergic diseases at mucous surfaces.

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